The photosynthetic response and extracellular carbohydrate production of tropical and temperate microphytobenthos

BY

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Statements and declaration

Declaration of originality

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Abbreviations

MPB - Microphytobenthos

PAM - Pulse Amplitude Modulated

SEM - Scanning Electron Microscopy

Chl. *a* - Chlorophyll a

TPTZ - 2,4,6-Tripyridyl-s-Triazine

HPLC - High Performance Liquid Chromatography

GCMS - Gas Chromatography Mass Spectrometry

RLCs - Rapid Light Curves

PAR - Photosynthetic activation radiation

F_v/F_m - Maximum photochemical efficiency

ETR - Electron transport rate

$rETR_{max}$ - Maximum relative electron transport rate

α - Initial slope of the relationship between PAR and rETR

E_k - Light saturation parameter

NPQ - Non-photochemical quenching

TCHO - Total extracellular carbohydrate

PCHO - Polysaccharide

MCHO - Monosaccharide

Acknowledgement

I would like to express my deepest gratitude to my primary supervisor Professor Andrew McMinn, for his continuous support of my PhD candidature, and the related research. I am also grateful for his patience, motivation, and immense knowledge that helped me throughout my candidature. His guidance, stimulating suggestions and encouragement helped me in all the time of research and writing this thesis. His devotion to research has influenced me deeply and encouraged me to continuously improving myself to be a better researcher – to think wiser, to work harder and to desire higher. I could not have imagined having a better supervisor than him.

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Abstract

Microphytobenthos (MPB) is a community of unicellular microorganisms that inhabit the photic zone of intertidal and subtidal areas of benthic zones. MPB contributes up to half of the total coastal primary productivity, as well as providing a major food source for many invertebrates, small fish and wading birds. Additionally, MPB plays an important role in stabilising the sediment by producing extracellular carbohydrates that form biofilms, which bind sediment particles together.

Intertidal zones experience extreme spatial and temporal fluctuations in both their physical, i.e. temperature, irradiances, and tidal position, and chemical, i.e. salinity and nutrient, conditions. MPB, therefore, needs to employ a variety of mechanisms to cope with these changes; these include behavioural changes, such as positioning themselves at the optimum position within the sediment, and physiological changes to their photosynthetic machinery. Extracellular polymeric substances (EPS) are one of the major components of the extracellular carbohydrates produced by MPB when they are undertaking these mechanisms. Much of this extracellular carbohydrate is produced for vertical migration but some is also produced as a product of photosynthetic overflow, when the MPB is experiencing photoinhibition.

This study focuses on the photosynthetic performance of MPB in different regions. As extracellular carbohydrate production and photosynthesis are often correlated, this relationship is examined in more detail here. A Pulse Amplitude Modulated (PAM) Fluorometer was used to measure the photosynthetic performance of the MPB, while several different approaches were used to investigate the extracellular carbohydrate production.

MPB species composition varies between regions and so the response mechanisms would also be expected to differ. The second chapter of this study focused on the investigation of the relationship between photosynthesis and extracellular carbohydrate production in a southern temperate region of greater Hobart area, Tasmania, at Penna Beach and Kings Beach, of different sediment grain size composition. The MPB was found to be well-adapted to its changing environment and did not experience photoinhibition during environment extremes, although its photosynthetic performance to change to cope with its changing environment. The performance varied between sampling sites and significant differences were found between biomass, photosynthetic performance, and extracellular carbohydrate production. Penna Beach was found to have significantly higher chlorophyll *a* (chl.*a*), extracellular carbohydrate

concentrations and photosynthetic performance than Kings Beach. The analysed parameters also varied significantly seasonally, with both chl.*a* and extracellular carbohydrate concentrations higher during spring and summer, while photosynthesis was higher during winter. Monosaccharides concentrations were found to be higher than polysaccharide concentrations at both sampling sites. However, there was no seasonal variations in mono- and polysaccharide concentrations.

The photosynthetic performance and carbohydrate production of MPB in a tropical region, Penang, Malaysia, was examined in Chapter 3. The chl.*a* and extracellular carbohydrate concentrations varied between sampling sites, where they were mainly affected by sediment temperature, salinity, and irradiance. Nitrate was the only nutrient that had a significant impact on the chl.*a* concentration and none of the major nutrient had a significant effects on the total carbohydrates (TCHO), monosaccharides (MCHO), or polysaccharides (PCHO). PCHO concentrations were significantly higher than TCHO at all sampling sites. Measured variable fluorescence showed that the MPB were not experiencing optimal conditions at any site, consistently having low photosynthetic efficiency (F_v/F_m). Unlike chl.*a* and extracellular carbohydrate, the photosynthetic performance was not affected by sediment temperature, salinity or irradiance. Photosynthesis was mostly affected by nutrient levels.

The colloidal monosaccharide component of carbohydrate was not able to be measured by the techniques used in earlier chapters (Chapters 2 and 3). In Chapter 4, however, using HPLC, the composition of the monosaccharide component was further differentiated into the key sugars, i.e. glucose, galactose, mannose, arabinose, rhamnose, and xylose. Glucose, which can be produced as photosynthetic overflow at high irradiances and low nutrients, was the major sugar in all samples at both sites. Furthermore, the glucose production varied significantly between sampling sites, with glucose concentrations at Penna Beach much higher (>0.1 mg/mL) than those of Kings Beach (<0.05 mg/mL). Glucose concentrations also varied significantly between seasons, being highest during spring and lowest during winter at Penna Beach, but this was not observed at Kings Beach. Additionally, the chl.*a* normalised glucose concentration did not vary between sampling depths at either site.

Real-time production of glucose was determined in the fifth chapter of this study, using an innovative experimental approach that adopted glucose biosensors to measure real-time changes in the concentration. Results showed that in natural communities, glucose production increased under high light and nutrient deficient conditions, although the single species cultures

had a more variable response. In a bacterial consumption experiment, glucose was rapidly consumed by bacteria in the dark. However, when the culture was treated with an antibiotic, consumption in the dark was minimal.

This study showed that MPB are able to rapidly adapt to changes in their environment. Extracellular carbohydrate, particularly glucose, was excreted mainly as a photosynthetic overflow product. Additionally, glucose biosensors have proven to be a reliable and innovative tool to measure the targeted sugar exudation in microphytobenthic biofilms and have considerable potential in future studies of MPB.

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Chapter 1: Introduction

1.1. Microphytobenthos (MPB)

Benthic microalgae, also known as microphytobenthos (MPB), are the photosynthetic microorganisms that inhabit the intertidal zones of coastal ecosystems and can contribute approximately 50% of total coastal primary production (Underwood and Kromkamp, 1999; Underwood and Provot, 2000). MPB supports life and plays a key role in maintaining biogeochemical processes in coastal ecosystems with previous studies showing that annual productivity in temperate intertidal mudflats is in the range of 10s to 100s C m^{-2} y^{-1} (Brotas and Catarino, 1995; Thornton *et al.*, 2002). However, due to anthropogenic activities in recent decades, there have been significant negative impacts on microphytobenthic ecosystems (Geider *et al.*, 1997).

Despite the important role of MPB to coastal foodwebs, few studies have documented their biomass at regional or global scales (Cahoon, 2006). Charpy-Roubaud and Sournia (1990) estimated global microphytobenthic production to be 0.34 Gt (10^9 metric tons) C yr^{-1} based on an average productivity estimate of 50 g C m^{-2} yr^{-1} for seawater ecosystems to depths of 50 m in coastal areas. Cahoon *et al.*(1999) calculated MPB production of up to 0.5 Gt C yr^{-1}, using the regionally- and depth-weighted production estimates from 108 studies. Most previous studies on microphytobenthic production and biomass were performed in estuarine and other shallow water (20 m depth) habitats at temperate latitudes (30° - 60°) (Cahoon, 2006). This comprises 85 studies in temperate water estuaries, 26 in European estuaries, 34 in the United States (U.S.) and Canadian estuaries, 19 in tropical (0° - 30° latitude) waters, and 6 in polar (60° - 90° latitude) waters (Cahoon, 2006) (Table 1.1.1). Furthermore, although data have been collected in temperate zones, they have typically focused on the northern temperate zones and southern temperate zones have been neglected, despite being equally important (Murphy *et al.*, 2004; Murphy *et al.*, 2008; Jordan *et al.*, 2008, 2010; Salleh and McMinn, 2011; Jackson *et al.*, 2010; Lee and McMinn, 2013; Maggi *et al.*, 2013).

Table 1.1.1. The average primary production of MPB in the previous 144 studies in different regions around the world (Cahoon, 2006).

Regions	Production (g C m^{-2} yr^{-1})
Temperate	50 – 100
European	97
U.S. and Canada	104
Tropical	527
Polar	24

MPB are made up of a diverse assemblage of phototrophic microalgae that are present within a few millimeters of the sediment surface, mainly dominated by diatoms (*Bacilariophyceae*), but including other microorganisms such as dinoflagellates (*Syndinniophyceae*), cyanobacteria and euglenoids (Guarini et al., 2000; Mitbavkar and Anil, 2004; Kromkamp *et al.*, 2006). The benthic diatoms are composed of 2 distinctive groups, epipsammic diatoms, which are closely attached to the sediment, and epipelic diatoms, which are free and motile in the sediment (Brotas and Catarino, 1995; Herlory *et al.*, 2004: Blanchard *et al.*, 2004). These unicellular microorganisms play important roles in coastal ecosystems and have a strong influence on nutrient flux within the sediments, as well as being a major food source for other trophic levels (Cahoon, 2006; Cook *et al.*, 2007).

Productivity within coastal ecosystems is primarily attributed to epipelic diatoms, which inhabit the cohesive sediments (Admiral *et al.*, 1984; Underwood and Paterson, 1993). Epipelic diatoms have a pennate frustule and raphe system (Round *et al.*, 1990), allowing them to migrate through the sediment. During periods of diurnal tidal emersion, cells migrate to the sediment surface, where they are able to photosynthesise (Taylor *et al.*, 1999; Staats *et al.*, 2000a). During immersion, when light intensity rapidly diminishes from attenuation by the usually turbid water column, cells migrate into the deeper sediment (Underwood and Paterson, 1993). A significant proportion of the carbon fixed by the MPB during photosynthesis is exuded into the surrounding environment, mainly as carbohydrates (Smith and Underwood, 1998; de Brouwer *et al.*, 2002; Bellinger *et al.*, 2005).

1.2. Microphytobenthic Extracellular carbohydrate production

Extracellular carbohydrates produced by microalgae have a significant influence on ecological processes in intertidal ecosystems. These exudates influence the microenvironment of biofilms by varying physicochemical parameters such as sediment porosity, density, absorption properties, hydrophobicity, and mechanical stability (Cahoon, 2006). This creates an equilibrium environment that favours MPB growth with respect to nutrients such as Ca^{2+}, nitrogen and phosphorous, and moisture which prevents cell desiccation (Decho and Lopez, 1993; Taylor and Paterson, 1998).

Although carbohydrate exudates contribute to the formation of biofilm mats when the environmental conditions favour diatom growth, the composition of extracellular carbohydrates varies among diatom species. The production of extracellular carbohydrates typically increases when sediment is exposed to the light (de Winder *et al.*, 1999; Taylor *et al.*, 1999; Staats *et al.*, 2000a); additional influences include length of exposure (Underwood and Paterson, 1993), algal growth stage (Staats *et al.*, 1999; Smith and Underwood, 2000), and mudflat morphology (Taylor and Paterson, 1998; Blanchard *et al.*, 2000). An increase in the production of extracellular carbohydrates has been observed at the start and end of the emersion period which also correlates with changes in diatom numbers at the mudflat surface (Smith and Underwood, 1998; Underwood and Smith, 1998). In diatom-rich biofilms, approximately 20 - 25% of the colloidal carbohydrate present is polymeric (Underwood *et al.*, 1995; Underwood and Smith, 1998). Therefore, up to 80% of the extracellular carbohydrates produced by epipelic diatoms consists of nonpolymeric material, mainly simple sugars, leachates, and other photoassimilates (Underwood *et al.*, 1995). Verdugo *et al.* (2004) estimated that there are 70 Pg of carbon as extracellular polymeric substances (EPS) in the ocean; a carbon pool that is considerably larger than the biomass of living organisms (1 to 2 Pg C (Falkowski *et al.*, 2000)). In intertidal zones, the extracellular carbohydrate production rates by MPB in sediments range from 0 to 18.2 μg glucose equivalents $(\mu g\ chl\ a)^{-1}\ h^{-1}$, or up to 1000 μg C $m^{-2}\ y^{-1}$ (Underwood and Paterson 2003).

Extracellular carbohydrates are categorised into two main fractions, colloidal and bound carbohydrates (Underwood and Smith, 1998; Blanchard *et al.*, 2000). Colloidal carbohydrates, also known as water-extractable carbohydrates, are comprised of glucose and low amounts of uronic acids. These are produced in the light and decomposed in the dark and often correlates with the concentration of the photosynthetic pigment chlorophyll *a* (Chl *a*) in intertidal

mudflats that are dominated by epipelic diatoms (Underwood and Smith 1998; Blanchard *et al.*, 2000). Bound carbohydrate, also known as EDTA-extractable carbohydrate, is a fraction of carbohydrate obtained via extraction with 100 nM EDTA (Ethylenediaminetetraacetic acid). This carbohydrate can only be recovered by using strong chelating agent and is rich in uronic acids and other charged groups that bind to the charged silt and clay particles though the bridging of divalent cations (Ca^{2+}, Mg^{2+}) (Underwood and Paterson, 1993; Blanchard *et al.*, 2000).

In intertidal zones, MPB biofilms serve as a major food source for other trophic levels (Hanlon *et al.*, 2006; Cook *et al.*, 2007). Sediment-dwelling invertebrates such as shellfish, polychaetes, and nematodes are largely dependent on the diverse assemblage of MPB. These invertebrates are in turn the food source for higher trophic levels such as wading birds and fish. Takai *et al.*, (2004) traced the flow of carbon through MPB, grazers, suspension feeders, up to carnivores using natural stable isotope abundance, clearly demonstrating the significant contribution of extracellular carbohydrates to trophic dynamics. With respect to the microbial loop, De Brouwer *et al.* (2005) determined that water-extractable carbohydrates are an important substrate for bacteria. The biofilms produced by MPB in surface sediments are highly labile pools of carbon that consist predominantly of glucose, which is preferentially degraded by bacteria (Giroldo et al., 2003; de Brouwer and Stal, 2001). Using ^{13}C as a trace, Middleburg *et al.* (2000) observed that carbon was rapidly transferred (within 1 hr) from MPB to bacteria and nematodes within the sediment at a site that was dominated by diatoms.

Extracellular carbohydrates can influence sediment stability as the bound carbohydrates contain various negatively charged groups such as uronic acids, sulfated sugars and ketal-linked pyruvate groups (Sutherland, 2001). These acidic carbohydrates may bind to the charged silt particles and effectively glue them together (Sutherland, 2001). Underwood and Paterson (1993) and de Brouwer *et al.*, (2002) showed that the presence of diatom biofilms is directly correlated to a reduction in sediment erosion. The formation of biofilm within the sediment further contributes to sediment stabilisation by reducing bottom roughness in the sediment (Paterson and Black, 1999). Paterson (1989) determined that the presence of motile epipelic diatoms stabilised intertidal sediments during emersion periods. Biofilm growth and sediment stabilisation is likely to be species-dependent. de Brouwer *et al.* (2005) showed that motile biofilms tend to detach shortly after secretion and are not likely to expand significantly along the shore. Other biofilms do extend along the shore, and these play a key role in mediating

sediment stabilisation, which in turn influences coastal morphology and the distribution of sediment within estuaries (de Brouwer *et al.*, 2005).

1.3. Benthic Ecosystems

Estuaries are a salient component of the world's coastal ecosystems as they support the economy of many coastal states by providing food and non-living resources, as well as influencing the local climate (Kromkamp *et al.*, 2006). Estuaries are typically comprised of intertidal mudflats, shallow subtidal sediments and sandbanks (Cahoon, 2006). These ecosystems are of considerable importance because together with the salt marshes and mangroves, they form natural barriers that provide the coast with protection from storm surge erosion (Kromkamp *et al.*, 2006). Hall (2000) suggested that changes in the status of these ecosystems would have detrimental economic and social consequences as well as impacting the welfare of the natural environment. Among the subsystems, the intertidal mudflats are important because they are highly productive due to both their shallow depth, which exposes benthic diatoms to higher irradiance, and relatively high nutrient (Kromkamp *et al.*, 2006).

1.4. The Environment and MPB

Most of the MPB are unicellular and are susceptible to environmental variability. Because intertidal ecosystems are highly dynamic habitats, MPB must adapted at both daily and seasonal scales. The main environmental drivers that influence photosynthesis are light (Pinckney and Zingmark, 1993; Barranguet *et al.*, 1998; Perkins *et al.*, 2001; 2010), sediment surface temperature (Blanchard *et al.*, 1997; Guarini *et al.*, 1997; Morris and Kromkamp, 2003; Yun *et al.*, 2010), sediment grain size (Paterson, 1989; Jesus *et al.*, 2009; Jesus *et al.*, 2005; Cartaxana *et al.*, 2006; 2016), CO_2 availability (Underwood and Kromkamp, 1999), and tidal amplitude (Miles and Sundback, 2000; Mitbavkar and Anil, 2004). Although nutrient concentration has a significant influence on photosynthesis in phytoplankton, the effect is not apparent on benthic microalgae. This reflects the high degree of *in situ* remineralisation by bacteria associated with biofilms and other nutrient inputs into estuaries (Underwood and Kromkamp, 1999). MPB in surface biofilms also respond to changing environment factors through behaviour mechanisms, such as vertical migration within the sediment (Kromkamp *et al*, 1998; Jordan *et al.*, 2008; Perkins *et al.*, 2010), and physiological mechanisms including induction of the xanthophyll cycle (Lavaud *et al.*, 2007; Perkins *et al.*, 2006; Jordan *et al.*, 2010).

Abiotic drivers are often correlated in the natural environment. For example, in the photic zone of muddy sediments, there is a positive correlation between median grain size and light penetration that is restricted to the top 500 μm (Jorgensen and des Marias, 1986; Cartaxana *et al.*, 2006). A positive linear correlation between light attenuation and sediment temperature has also been observed, which infers an indirect relationship between the sediment grain size and the sediment temperature (Guarini *et al.*, 1997). Other co-variates include tidal position, sediment type and irradiance (Mitbavkar and Anil, 2004; Jesus *et al.*, 2009); photosynthetic activity is relatively high at midday during low tide when cells are mostly at the surface of the sediment and wave impact is minimal (Brotas and Catarino, 1995; Mitbavkar and Anil, 2004).

1.4.1. Sediment temperature

Sediment temperature is one of the main drivers of MPB performance in the intertidal (Barranguet et al., 1998; Morris and Kromkamp, 2003; van Leeuwe et al, 2008; Yun et al., 2010; Du et al., 2012; Lee and McMinn, 2013). MPB increase their vertical migration when sediment temperature increases, and this movement is typically away from the sediment surface (Du et al., 2012).

The responses of MPB in the sediment are species-specific - different diatom species have different surface area to volume (SA/*V*) ratios due to their overall size (Yun *et al.,* 2010). These ratios can provide insight into both ecological and physiological activity for unicellular algae with respect to growth rates, nutrient uptake, and sinking rates. MPB species with large SA/*V* ratios are essentially considered to have an advantage under optimal environmental conditions, where irradiances, temperature and other environmental variables are most favourable. This morphology enhances the uptake efficiency of diluted inorganic nutrients (Harris, 1986). Having said that, smaller MPB species exhibit a higher light harvesting efficiency (Raven 1998). Sumich (1984) observed lower sinking rates with increased SA/*V* ratios in phytoplankton, which increased residence time in the upper, sunlit waters. Conversely, Yun *et al.* (2010) described the opposite trend when diatoms were acclimated to high temperature and/or strong irradiance. Large MPB species with smaller SA/*V* ratios showed a greater resistance to high temperature. As a result, these MPB are able to inhabit the sediment surface at midday when the temperature is high. Because smaller MPB have higher SA/*V* ratios, they are vulnerable to high temperature and are more likely to migrate down to the sediment sub-surface to prevent photodamage. Yun *et al.*, (2010) also determined that only benthic diatoms with very small SA/*V* ratios are able to undergo photosynthesis at very high temperatures (Fig.

1.4.1.1). Morris and Kromkamp (2003) documented the response of five benthic diatoms to elevated temperature (up to 35°C) and suggested that high temperature tolerance would be typical of most algae with small SA/*V* ratios (Fig. 1.4.1.2).

Temperature also influences processes such as diffusion and cellular pH. Similarly, denaturation of chlorophyll-proteins and inactivation of oxygen-evolving mechanisms can be triggered by temperature extremes (Briantais *et al.*, 1996). For example, Morris and Kromkamp (2003) observed the complete abolition of oxygen evolution and variable fluorescence in *Cylindrotheca closterium* at 40°C. In another study, van Leeuwe *et al.* (2008) found that high irradiance and de-epoxidation (a reversible process of conversion from diadinoxanthin (DD) to diatoxanthin (DT)) was seasonally variable. The most likely explanation for this is that the xanthophyll pigment cycle was affected by temperature. These authors proposed that amplification in the de-epoxidation state of MPB during winter is due to the build-up of excess energy to protect against photoinhibition. This idea is supported by Serôdio *et al.* (2005) who demonstrated that MPB exhibited higher non-photochemical quenching (NPQ) (dissipation of excess heat energy through xanthophyll cycle) during seasonal periods characterised by low temperature/high irradiance.

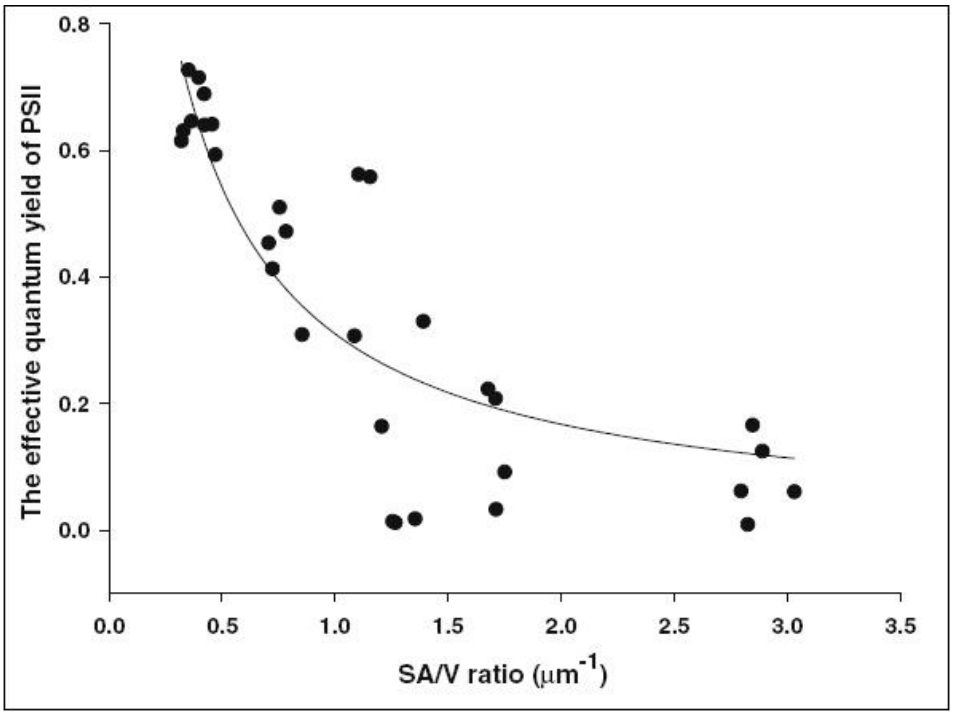
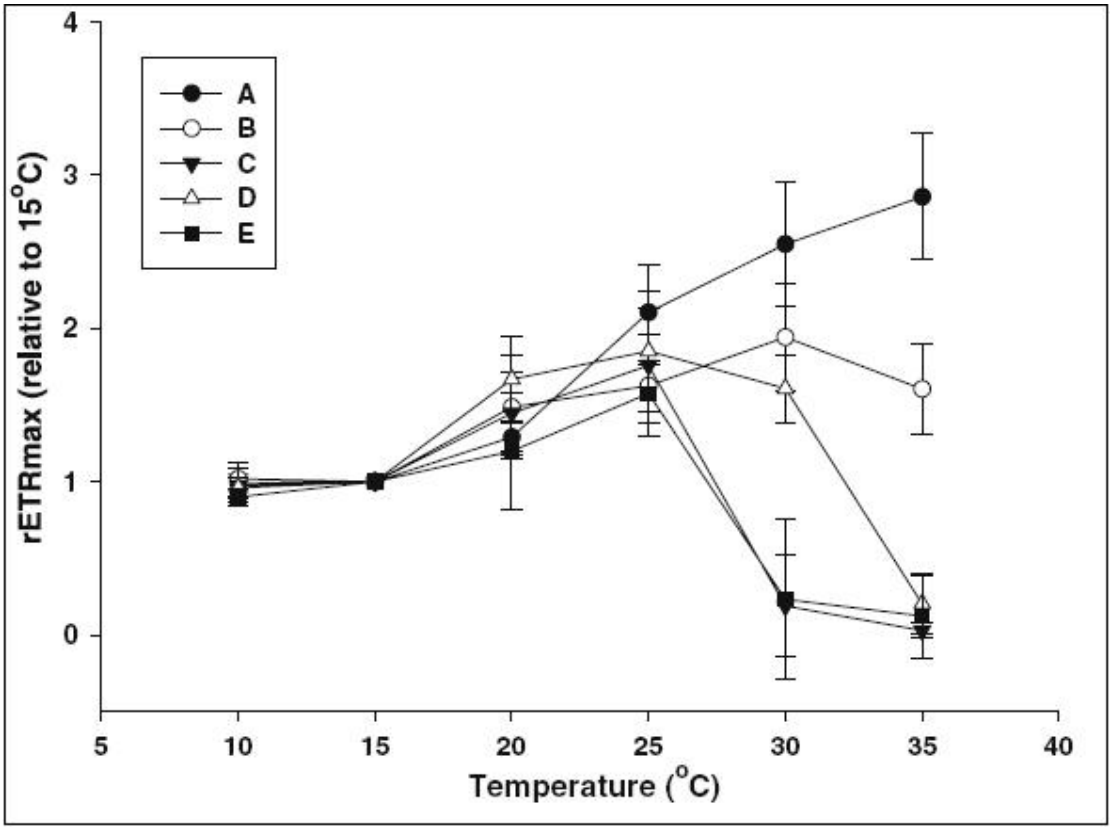


Figure 1.4.1.1. Relationship between SA/*V* ratios and the effective quantum yield of PSII at the same irradiances as the growth irradiance (100 μmol photons $m^{-2}s^{-1}$) in samples exposed to

195 a temperature of 35 °C. The equation of the curve is y-a (1 + *h* X χ), (R^2= 0.76, P < 0.01) (Yun
196 *et al.*, 2010).



197

198 Figure 1.4.1.2. Relative effect of temperature on $rETR_{max}$: *P. elongatum* (A), *A. coffeaeformis*
199 (B), *C. closterium* (C), *Navicula* sp. (D), and *Nitzschia* sp. (E). All parameters were normalized
200 to the values at 15°C where they were used to be grown at. Data represent means and standard
201 deviations (Yun *et al.*, 2010).

202 **1.4.2. Light intensity**

203 MPB biofilms are exposed to variable light intensity during tidal cycles (Chevalier *et al.*, 2010).
204 These conditions can change the composition of microalgael communities at the sediment
205 surface and the behaviour of epipelic diatoms with respect to *in situ* migration (Perkins *et al.*,
206 2001). Photosynthetic performance can also be highly variable. The parameter $rETR_{max}$, which
207 describes the flow of electrons from PSII to PSI, has been used effectively to describe tidal-
208 driven stress in MPB biofilms, where $rETR_{max}$ is lower during midday when irradiance is
209 relatively higher (Chevalier *et al.*, 2010) (Fig. 1.4.2.1). Because episammic microalgae lack
210 the ability to migrate deeper into the sediment, they adjust physiologically to cope with the
211 damaging light, by developing photoprotection mechanisms such as the xanthophyll cycle.

This is in contrast to epipelic microalgae which can both migrate further down in the sediment, as well as adjust physiologically as photo-protective measure (Cartaxana *et al.*, 2016; Serôdio *et al.*, 2012).

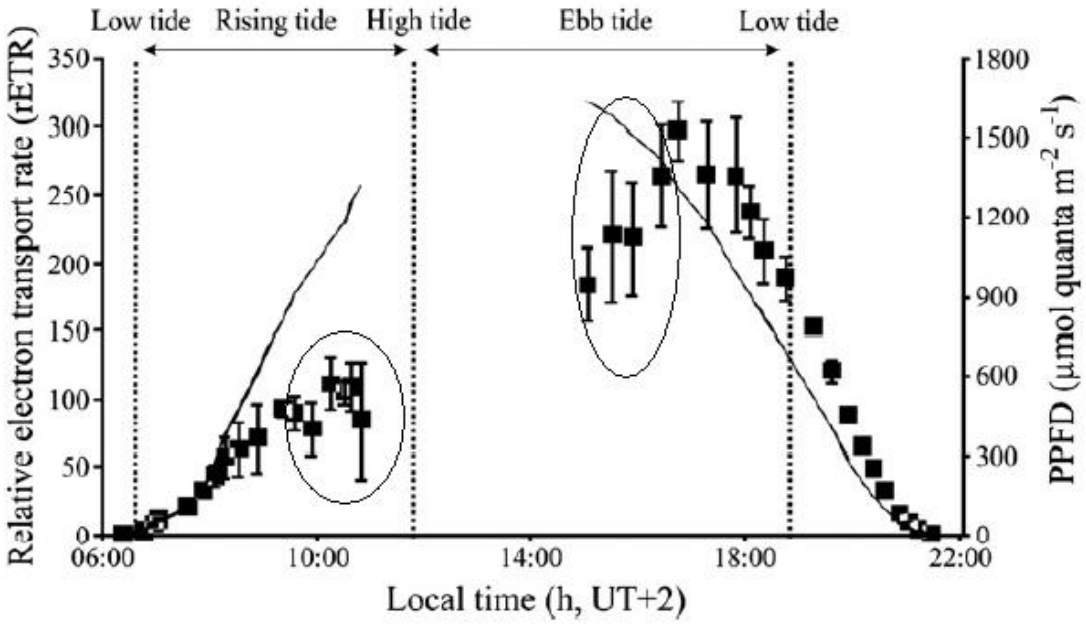
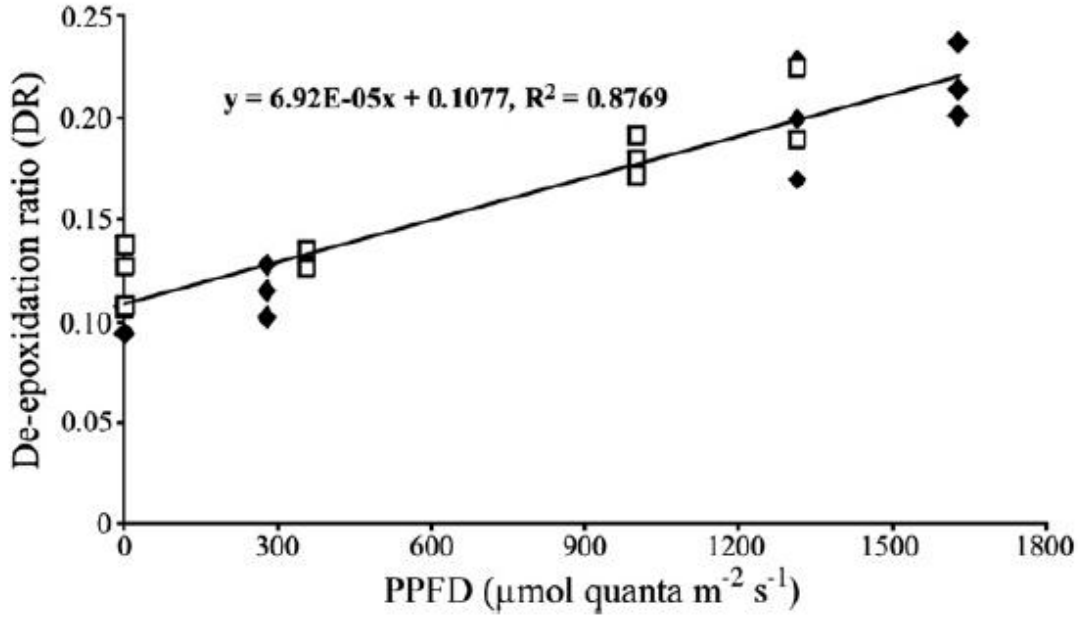


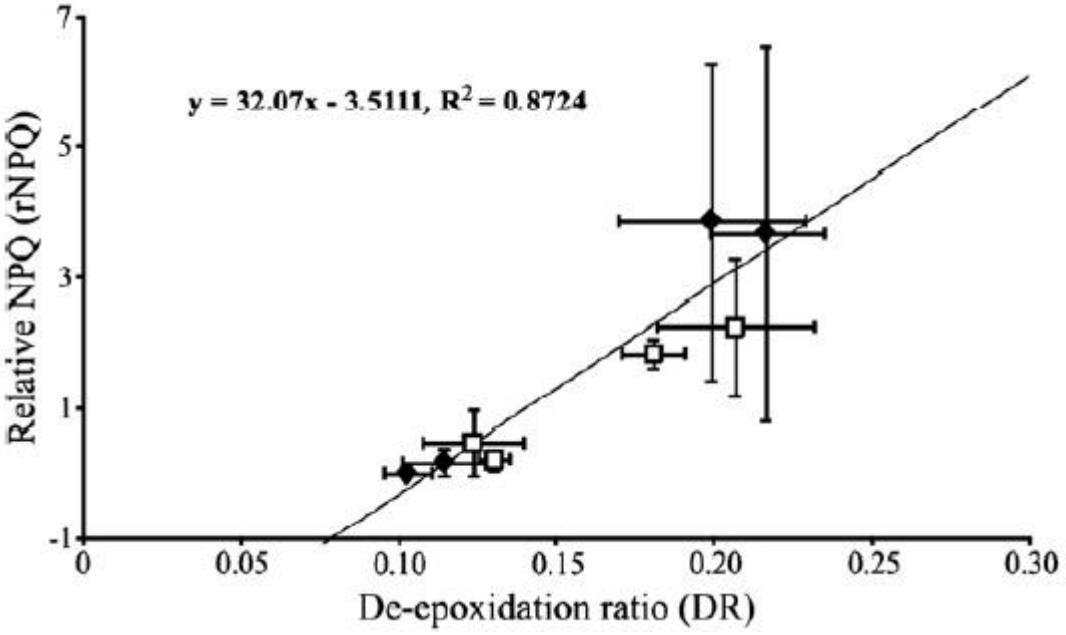
Figure 1.4.2.1. Diurnal course of mean relative electron transport rate (rETR, ■ and vertical bars: standard deviation (SD)) measured on three spots on the sediment and incident irradiance (PPFD, solid line) during the tidal cycle. Circles showed low rETR during midday (Chevalier *et al.*, 2010).

Chevalier *et al.* (2010) also demonstrated that xanthophyll cycling occurred *in situ* as the de-epoxidation ratio (DR) was correlated to changes in irradiance (Fig. 1.4.2.2) and that relative NPQ is positively correlated to DR during the whole day (Chevalier *et al.,* 2010) (Fig. 1.4.2.3). These observations are important in illustrating the role of the xanthophyll cycle in cells exposed to high light intensity (Lavaud *et al.*, 2004). The *in situ* study of MPB under extreme conditions is therefore considered important for determining how these microalgae respond to highly dynamic environments.



227

228 Figure 1.4.2.2. De-epoxidation ratio (DR) as a function of incident irradiance (PPFD) for the
229 morning emersion period (am, □) and the afternoon emersion period (pm, ■. Solid line: linear
230 regression (Chevalier *et al.* 2010).



231

232 Figure 1.4.2.3. relative NPQ (rNPQ) as a function of mean de-epoxidation ratio (DR) for the
233 morning emersion period (□) and the afternoon emersion period (■) (horizontal and vertical
234 bars: SD). Solid line: linear regression (Chevalier *et al.* 2010).

1.4.3. Sediment Type

Sediment type plays an equally important role in shaping both the taxonomy of MPB assemblages as well as photo-regulation strategies (Jesus *et al*., 2009). For this reason, a comparison between muddy and sandy environments is essential. Muddy and sandy environments represent very different substrates for MPB and these influence the taxonomic abundance of a range of microbial taxa (Cartaxana *et al.*, 2006; Jesus *et al.*, 2009). Jesus *et al.* (2009) showed that the muddy sites were mostly dominated by diatoms while sandy sites were composed of mixed algal populations including diatoms, euglenids and cyanobacteria. Additionally, Jesus *et al.* (2009) determined that cyanobacteria dominated sandy sites during summer when the temperature was high, while diatoms were present within the sediment throughout the year.

Sediment also plays a role in structuring the vertical distribution of MPB communities. In mud, cells are restricted to several millimetres, while cells present in sand typically occupy several centimetres (Jesus *et al.*, 2009). Differences in community composition between sediment types can be largely attributed to light attenuation. Significantly higher DT/DD ratios (ratio of photoprotective pigments) have been measured in subsurface sediments (Fig. 1.4.3.1), which infers that xanthophyll cycling was undertaken by MPB for down regulation in direct response to variation in light climate (Jesus *et al.*, 2009). This process is thought to reflect the production of photoprotective pigments by epipsammic MPB. Conversely, in the same study, Jesus *et al.* (2009) found that the epipelic MPB did not show any changes in DT/DD ratios with depth; a response that illustrates that these species are low-light acclimated and are capable of vertical migration. Importantly, Serôdio *et al.* (2005) have shown that epipelic MPB are able to maintain highly flexible photoacclimation mechanisms under low light.

1.5. Glucose biosensors analysis

Various methods are available to quantify extracellular carbohydrates, but most techniques are destructive (i.e. destroy the cell). In addition, these methods do not measure instantaneous production and intracellular changes are highly likely during the time period prior to laboratory analysis. To overcome this issue, I collaborated with Pinnacle Technology (USA) on the design of a modified glucose biosensor.

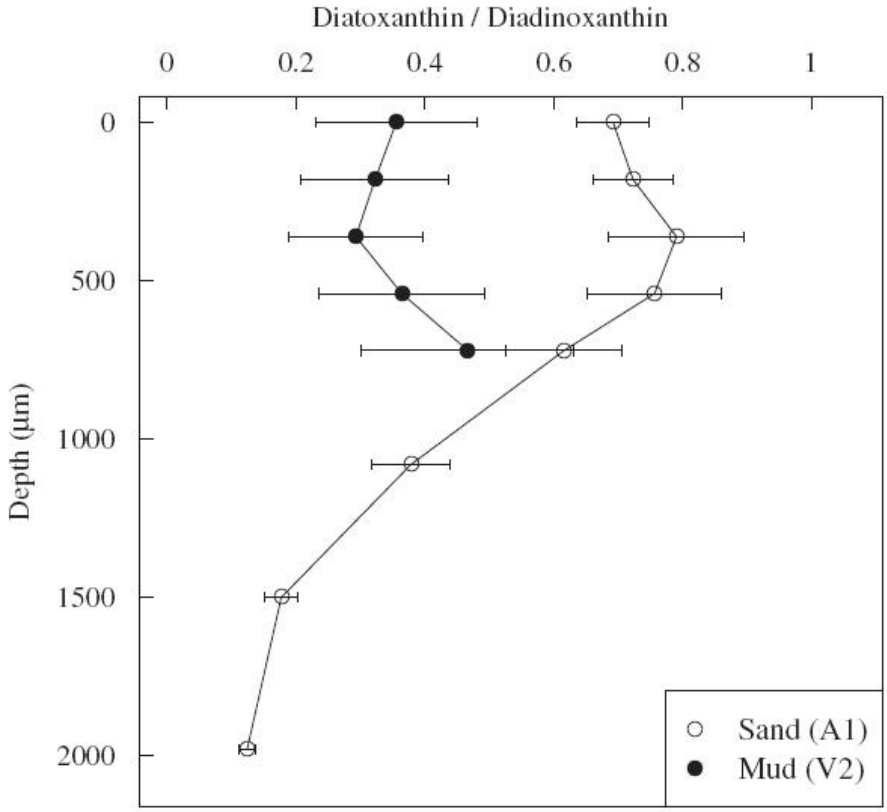


Figure 1.4.3.1. Depth profile of the diatoxanthin-diadinoxanthin (DT/DD) ratios. A1 and V2. A1 is plotted to 2 mm depth and V2 only to 750 mm. Mean ± SE of all sampling dates (Jesus *et al*., 2009).

This biosensor uses the enzyme glucose oxidase to reduce glucose and produce hydrogen peroxide as a by-product. The hydrogen peroxide is reduced on a platinum electrode to produce electrons that are then detected on an Ag/AgCl reference electrode (Fig. 1.5.1). Because previous studies have demonstrated that MPB produce glucose as the main product of photosynthetic overflow under stress conditions (Mueller et al., 2016; Cook et al., 2007; Maranon et al., 2004), hence I propose that glucose concentrations can be used as a proxy to determine stress in MPB. This will be the first study that provides instantaneous glucose concentrations in the benthic environment.

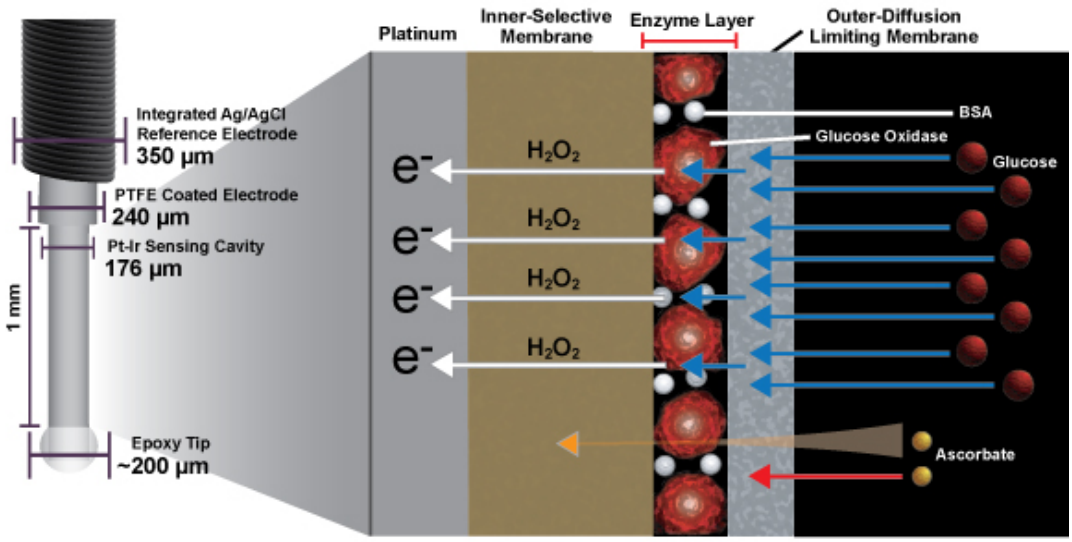


Figure 1.5.1. Diagram showing how the biosensor operate. Reprinted from Glucose biosensors, by Pinnacle Technology Inc, 2015, retrieved from (http://www.pinnaclet.com/glucose.html). Copyright (2015) by Pinnacle Technology.

1.6. Hypotheses and Aims

In this thesis, I investigate the performance of MPB within the intertidal zone at different spatial and temporal scales. The research will start with the study of general MPB photosynthesis and extracellular carbohydrate production at Southern Temperate Regions. The maximum photochemical efficiency (F_v/F_m), maximum relative electron transfer rate ($rETR_{max}$), initial slope of Rapid Light Curve (RLC) (α), and light saturation parameter (E_k) are measured with chlorophyll fluorescence method, while the extracellular carbohydrate production is measured with 2, 4, 6-Tripyridyl-s-Triazine (TPTZ). Same approaches are carried out to study the MPB from Tropical Region in order to gain better understanding of MPB from different regions. As I am most interested in the production of extracellular carbohydrate, particularly glucose, I will be introducing the cutting-edge technology of using the glucose biosensor to investigate the real-time production of glucose by MPB *in-vitro*. The study is then followed by qualitatively studying the extracellular carbohydrate produced by MPB. This is achieved by using the High-Performance Liquid Chromatography (HPLC) analysis.

The overarching questions and aims addressed in this thesis are as follows:

Chapter 2:

Question: What is the temporal (fortnightly, monthly, seasonally) response, i.e. photosynthesis and extracellular carbohydrate production of MPB to environmental stress?

Hypothesis: MPB response to their changing environment by altering their photosynthesis, or micromigration to avoid photodamage on them.

Hypothesis: MPB produce extracellular carbohydrate as well as photosynthetic overflow when they are experiencing environmental conditions exceeding the threshold they could withstand.

Chapter 3:

Question: Do MPB in coastal zones of Tasmania and Peninsular Malaysia similarly to each other?

Hypothesis: MPB of tropical country perform the response mechanisms similarly to those of Southern Temperate Region in response to their environment, although it is species-specific.

Chapter 4:

Question: How do MPB respond to environmental stress simulated in the laboratory?

Hypothesis: F_v/F_m of MPB decreases under high sediment temperature, as well as nutrient depleted condition.

Hypothesis: Glucose is produced as photosynthetic overflow in response to the stressed condition.

Hypothesis: Glucose is consumed rapidly by bacteria.

Chapter 5: Do concentrations of microphytobenthic extracellular carbohydrate vary between depths in the Southern Temperate Regions?

Hypothesis: There are different concentrations of extracellular carbohydrate in the sediment, where they are produced, as well as being consumed at the same time.

Chapter 2: The extracellular carbohydrate production and photosynthetic performance of microphytobenthos in the southern temperate region (Hobart, Tasmania) in response to various environmental variables

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2.1. Abstract

Intertidal flats are highly dynamic in their responses to a changing environment and hold important role in contributing to the total coastal productivity. This is largely due to benthic microalgae, called microphytobenthos (MPB) that inhabit these coastal areas. MPB consist of unicellular microorganisms and are mainly composed of diatoms, dinoflagellates, euglenoids, and relatively amounts of cyanobacteria, as well as photosynthetic bacteria. MPB can respond both behaviourally and physiologically to changes in their environment, and these responses are closely coupled to the extracellular carbohydrate production. Extracellular carbohydrates are produced during the MPB vertical micro-migration within the sediment, an MPB mechanism to position themselves in the optimum depth for photosynthesis, as well as to move away from photo-damaging irradiance. Extracellular carbohydrates are also produced as photosynthetic overflow when MPB are experiencing limiting nutrient conditions. In some occasions, extracellular carbohydrates are excreted as an excessive product when MPB are under optimal condition. This study aims to determine the relationship between MPB performance and their extracellular production in response to different environmental variables in southern temperate regions (Tasmania). Samples were collected bi-weekly at Penna Beach (42° 47’ 06"S, 147° 31’ 13"E) and Kings Beach (42° 53’ 44"S, 147° 20’ 01"E) of the greater Hobart area. Five replicates of sediment samples were collected for biomass and PAM analyses using a 45-mm diameter core. Another three replicates of sediment samples were collected to determine the concentrations of extracellular carbohydrates. The samples were extracted with MQ water and the concentrations of extracellular carbohydrate were determined spectrophotometrically. Total carbohydrate concentrations were further measured spectrophotometrically on the hydrolysed samples and polysaccharide concentrations were then obtained by the subtracting the values of hydrolysed samples from the values of samples prior hydrolysing.

Significant differences (P-values < 0.05) were found between sampling sites regarding the biomass and extracellular carbohydrate production, where Penna Beach had significant higher chlorophyll and extracellular carbohydrate concentrations, and photosynthetic performance than those of Kings Beach. The analysed parameters varied significantly seasonally, where both chlorophyll and extracellular carbohydrate concentrations were higher during spring and summer, while photosynthesis was higher during winter. Monosaccharides concentrations were relatively higher than polysaccharide concentrations at both sampling sites. However, no

seasonal variations for both mono- and polysaccharides were observed within both sampling sites.

MPB are able to respond rapidly to their changing environment, through different mechanisms. Extracellular carbohydrate production is part of most of the response mechanisms employed by MPB. In this study, elevated monosaccharide production indicated that these carbohydrates were produced as photosynthetic overflow in response to extreme environmental conditions. Additionally, the increased monosaccharide concentrations were likely due to the breaking down of larger sugar components into monosaccharide through hydrolysis via bacterial breakdown.

2.2. Introduction

Microphytobenthos (MPB), also known as benthic microalgae, are photosynthetic microorganisms that inhabit in photic benthic zones of coastal ecosystems. Microphytobenthic communities are vital components of most shallow marine ecosystems, contributing between 20% and 50% of annual primary production (Underwood and Provot, 2000; Blanchard *et al.*, 2002; Herlory *et al.*, 2004; Cahoon *et al.*, 1999). MPB assemblages are usually dominated by diatoms and mostly concentrated in shallow water environments, where they are exposed to highly variable environmental conditions.

The intertidal area is one of the most important components of coastal ecosystems, as it is a highly productive area that fringes relatively large feeding grounds for higher trophic organisms such as birds, fishes and shellfish. Intertidal areas are highly dynamic and affected by various environmental variables, such as irradiance (Serôdio *et al.*, 2005; 2006; Du *et al.*, 2010; Perkins *et al.*, 2010; Cartaxana *et al.*, 2013), temperature (Morris and Kromkamp, 2003; Defew *et al.*, 2004; Hancke *et al.*, 2008; Yun *et al.*, 2010; Vieira *et al.*, 2013), sediment grain size (Jesus *et al.*, 2006; Cartaxana *et al.*, 2006; Jesus *et al.*, 2009), tidal amplitude (Miles and Sundback, 2000; Mitbavkar and Anil, 2004), nutrients (Brotas and Catarino, 1995; Sin *et al.*, 2009; Fonseca *et al.*, 2013). These variables can change both spatially and temporally both at small and large scales.

Owing to their highly dynamic environments, MPB are well-adapted in order to survive in their habitats. MPB carry out different mechanisms in response to the changing environment, behaviourally and physiologically, e.g., by migrating within the sediment and changing their photosynthetic performance, respectively (Lee and McMinn, 2013; Perkins *et al.*, 2010; Jesus *et al.*, 2009; Mouget *et al.*, 2008). In order to position themselves in the optimum position for photosynthesis or preventing photo-damage during migration, MPB excrete extracellular carbohydrates to assist in vertical movement, as well as to position themselves to the sediment grain. Previous studies showed that MPB migrated into the subsurface of the sediment when the surface conditions were unfavourable (Serôdio and Catarino, 2000; Blanchard *et al.*, 2001, 2002, 2004; Jesus *et al.*, 2006). Bellinger *et al.* (2010) also stated that the production of extracellular carbohydrate production is typically greater when the environment is nutrient-limited, with an increased need for photosynthetic overflow mechanisms in the form of carbohydrates to protect the photosystems from damage. On the other hand, MPB can respond to its changing environment by altering its photosynthetic performance, i.e. changing the rates

401 of the processes in its photosystems (Cartaxana *et al.*, 2013; 2016; Chevalier *et al.*, 2010;
402 Jordan *et al.*, 2010; Serôdio *et al.*, 2008).

403 Extracellular carbohydrates play a significant role in microphytobenthic communities, as well
404 as in entire coastal ecosystems (Hardison *et al.*, 2013; de Brouwer *et al.*, 2005; Underwood and
405 Paterson, 2003; Fleming and Wingender, 2002; Smith and Underwood, 1998). The production
406 of extracellular carbohydrates improves the sediment stabilisation in the coastal zones.
407 Additionally, microphytobenthic biofilms are protected from desiccation when they are
408 exposed to the environment by the highly moisture environment from the extracellular
409 carbohydrates (Cahoon, 2006). Under certain circumstances, extracellular carbohydrates can
410 also be produced as photosynthetic overflow products, when environmental conditions are at
411 optimum (Perkins *et al.*, 2001). Hence, extracellular carbohydrate production is not solely due
412 to unfavourable environmental stressors, which requires more sophisticated investigations,
413 which take the relationship between the photosynthetic performance, extracellular
414 carbohydrate production, and chlorophyll production into account.

415 In Tasmania, microphytobenthic assemblages experience relatively large variations in their
416 surrounding environment seasonally, because temperature and irradiance can change
417 dramatically between summer and winter. Hence, this study aims to determine the production
418 of microphytobenthic communities of Southern Temperate Region, Tasmania, in terms of
419 photosynthetic performance and carbohydrate production, the relationships between these
420 observations, and their responses to different environmental variables, and investigates the
421 hypothesis that extreme environmental conditions in both physical and chemical properties
422 provide stresses on the community and affect their performance. Investigations were carried
423 out in the field throughout a year in order to gain understanding on how these unicellular
424 microorganisms respond to different environments over a full seasonal cycle.

2.3. Materials and Methods

2.3.1. Sampling Sites

This study was undertaken at two sampling sites, Penna Beach (42° 47’ 06"S, 147° 31’ 13"E) in Pittwater Reserve area and Kings Beach (42° 53’ 44"S, 147° 20’ 01"E) in Errol Flynn Reserve, Sandy Bay (Fig. 2.3.1). These sampling sites were chosen due to their variation in physical sediment properties, where Penna Beach has a finer grain size compared to Kings Beach (Fig. 2.3.2).

Figure 2.3.1. Sampling sites that were chosen in the greater Hobart Area, Tasmania for the current study.

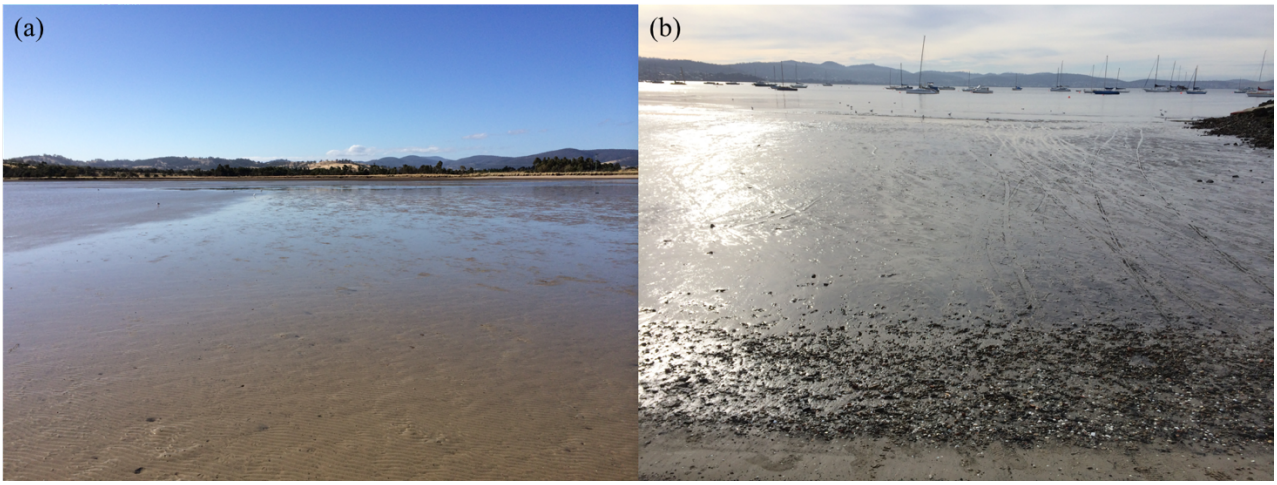


Figure 2.3.2. Photographs of sampling sites during low-tide period, where (a) is Penna Beach at Sorell and (b) is Kings Beach at Sandy Bay.

2.3.2. Physical and chemical properties of the study sites

The irradiance and salinity at each sampling site were measured during each sampling day using a Biospherical QP radiometer with a 2π sensor and an ATAGO E-Line refractometer (ATC Range, ATAGO CO. Ltd., Japan), respectively. Vertical temperature profiles within the sediment were measured by inserting a temperature probe (HI 766C) (K-type thermocouple thermometer, HI935005, Hanna Instruments, Woonsocket, Rhode Island, USA) vertically into the sediment in 1 mm intervals, down to 20 mm. Sediment grain size was analysed at both sampling sites using standard sieving methods (Folk, 1974). Monthly nutrient levels were obtained from The Derwent Estuary Program (DEP), including nitrite and nitrate (NO_x), dissolved reactive phosphate (DRP), and ammonia and ammonium (NH_4) concentrations (Coughanowr *et al.*, 2015).

In addition, relative species abundance was determined at both sampling sites, by collecting the top 1 cm sediments using a 45 mm-diameter corer. A preliminary count was undertaken on another collected fresh samples in order to determine that most of the cells observed are comprised of living cells. Then, samples were preserved in Lugol's iodine solution prior to preparation for Scanning Electron Microscopy (SEM) analysis. Hydrogen peroxide (H_2O_2) was used to clean the preserved samples. The samples were filtered through 2 m membrane filters and mounted on the SEM stubs with double adhesive tape. The stubs were examined using a Hitachi SU-70 Analytical Field Emission Scanning Electron Microscope (FESEM) (Hitachi,

Naka Division, Ibaraki Prefecture) and species identification was carried out following Saunders *et al.* (2010) and MPB relative abundance was determined (n= 200).

2.3.3. Sampling protocols

Sampling was carried out fortnightly at both sampling sites during spring tides at the low tide periods from February 2014 to February 2015. Two periods were chosen during each sampling date, on the beginning and the end of low tide periods, when the intertidal zones were just starting to expose and before they immersed, respectively. This approach was to determine the MPB performance i.e. photosynthesis and extracellular carbohydrate production before and after the low tide period. A 45 mm-diameter corer was to collect the sediment samples for the analysis. Collected samples were sliced into 1 mm intervals at the top 3 mm of the sediment for further analyses.

2.3.4. Chlorophyll analysis

The chlorophyll was extracted with 10 mL of methanol for 8 hours upon returning to the laboratory and measured on a Turner Designs 10AU fluorometer (Sunnyvale, CA, USA) using the acidification method (Holm-Hansen *et al.*, 1965). The fluorometer was calibrated against a chlorophyll a standard (Sigma-Aldrich Chemical Co., St Louis, MO, USA).

2.3.5 Chlorophyll a fluorescence measurements and Rapid Light Curves

Five replicate samples were measured for chlorophyll fluorescence using a high-resolution Pulse Amplitude Modulated fluorometer (Water PAM; Walz, Effeltrich, Germany) in the field according to Lee and McMinn (2013). In short, sectioned samples were mixed with 10 mL filtered seawater and left in the dark for at least 30 minutes for dark-adaptation. After that, the photosynthetic performance of dark-adapted samples was measured using the Water-PAM and Rapid Light Curves (RLCs) were obtained via a light treatment consisting of a saturating pulse of light followed by eight consecutive 10 s intervals of increasing actinic light of 0, 105, 162, 242, 346, 484, 632, 980, and 1351 μmol photon $m^{-2}s^{-1}$. Different photosynthetic parameters were acquired, following the methods and formulas provided by Ralph and Gademann (2005) (Table 2.3.1):

$$rETR = rETR_{max} [1 - \exp (-\alpha Ed/rETR_{max}] \quad \text{…… (Equation 1)}$$

$$E_k = ETR_{max}/\alpha \quad \text{…… (Equation 2)}$$

Where $rETR_{max}$ represents the maximum potential rETR in the absence of photoinhibition, α is the initial slope of the light curve before the onset of saturation and represents the efficiency of light utilization, and E_d is the irradiance (in the general formula 400 – 700 nm).

Table 2.3.1. Parameters and definitions of abbreviations used in this study

Parameters	Definition	Units
α	Initial slope of the relationship between PAR and rETR	Dimensionless
E_k	Light saturation parameter	μmol photon $m^{-2}s^{-1}$
F_v/F_m	Maximum photochemical efficiency	Dimensionless
$rETR_{max}$	Maximum relative electron transport rate	Dimensionless
PAR	Photosynthetic active radiation	μmol photon $m^{-2}s^{-1}$

2.3.6. Extracellular carbohydrate analysis

Three replicate sampling cores were sampled for extracellular carbohydrate analysis. The top 1 mm sediments were sectioned and kept in the ice box filled with dry ice in order to prevent changes in concentrations of the extracellular carbohydrate. Extraction methods were undertaken upon returning to the laboratory as described by de Brouwer et al. (2002). In short, the water-extractable, i.e., bound-extracellular carbohydrate fraction was obtained by extraction of the sediment with MQ water at 30 °C for an hour. Subsequently, extracellular carbohydrate contents were extracted by the extraction of the sediment with 0.1 M Na_2 EDTA for 16 hours at room temperature, and this is referred to as the colloidal-extracellular carbohydrate fraction.

Monosaccharide (MCHO) fractions were obtained from both these extraction alternatives. Extracted samples were stored at -20 °C in a freezer until further analysis. Samples were hydrolysed prior to spectrophotometric analysis according to Myklestad et al. (1997). In short, 4 mL of extracted samples and 0.4 mL of 1 M HCl were added to 5 mL glass ampoules. The sealed ampoules were placed in a heat chamber at 100 °C for 20 hours. After the hydrolysis was completed, samples were taken out from the heat chamber and cooled before opening. 1

M NaOH was added to each ampoule to neutralise the mixture in the ampoule. Hydrolysis provides information of total extracellular carbohydrate (TCHO) concentrations in the sediment samples. Prior to analyses, all glassware was acid washed with 10% HCl for at least 2 hours and rinsed >3 times with MQ water, to ensure that all equipment was scrupulously clean.

Carbohydrate compositions of bound- and colloidal-extracellular carbohydrates were measured spectrophotometrically based on protocols developed by Myklestad et al. (1997) and modified by Hung and Santschi (2001). In brief, 1 mL of extracted sample was mixed with 1 mL of Potassium Ferricyanide and incubated in boiling water for 10 minutes. The mixture was then immediately spiked with 1 mL Ferric Chloride and 2 mL 2,4,6-Tripyridyl-s-Triazine (TPTZ) and mixed well in a Vortex mixer. After 30 minutes, the absorbance of the samples was determined at 595 nm with a Cintral UV/Vis spectrophotometer (PerkinElmer, Waltham, Massachusetts, USA) against distilled water. The analysis was carried out in the dark or with minimal red-light due to the high light sensitivity of the analytical reagents (van Oijen et al., 2003). The concentrations of polysaccharide (PCHO) was calculated by subtracting the concentration of MCHO from TCHO. The units were expressed as μmol CL^{-1}, i.e. μmoles of glucose-C per litre and was converted to μg glucose-C per litre by multiplying μmol CL^{-1} by 30 and normalised to chl-*a* concentrations (mg C [mg chl. a]$^{-1}$).

2.3.7. Statistical analysis

Measured data were compiled in Microsoft Excel and statistical analyses were performed with computing software, R (R-Core-Team, 2014). Analysis of Covariate (ANCOVA) was carried out to determine the relationship between the observed data and the environmental variables. Additionally, Analysis of Variance (ANOVA) was undertaken to study the most significant variation on the microphytobenthic photosynthetic parameters and extracellular carbohydrate production, both temporally and spatially. The significance level of P-value 0.05 was considered to be significant.

2.4. Results

2.4.1. Environmental Data

The daily irradiance was highest during summer and lowest during winter at both sampling sites, with 2210 µmol photons $m^{-2}s^{-1}$ and 460 µmol photons $m^{-2}s^{-1}$ at Penna Beach, and 2140 µmol photons $m^{-2}s^{-1}$ and 410 µmol photons $m^{-2}s^{-1}$ at Kings Beach, respectively. Sediment surface temperatures often correlated with the irradiance, hence highest sediment surface temperatures observed at Penna Beach were 26.12 °C ± 0.17 during early emersion period and 29.14 °C ± 0.10 during the late emersion period on 29th December 2014, and those of Kings Beach were 26.92 °C ± 0.33 during the early emersion period on 5th January 2015 and 24.26 °C ± 0.08 during late emersion period on 2nd January 2015.

Salinity showed a strong relationship with precipitation; therefore, the highest and lowest salinities were observed at both sampling sites, during summer when the rainfall was minimum and during winter when rainfall occurred more frequently, respectively (Table 2.4.1). However, there was a time lag for the salinity to decrease after rainfall, as it requires time for the freshwater input to mix well with the surface seawater at the sampling sites. This was observed for the salinity values acquired on 17th and 19th August 2014, where there was 17.6 and 10.2 mm of rainfall on 1st and 2nd August, respectively. This shows that at these sampling sites it took approximately 2 weeks for the freshwater input to alter the salinity at the sampling sites.

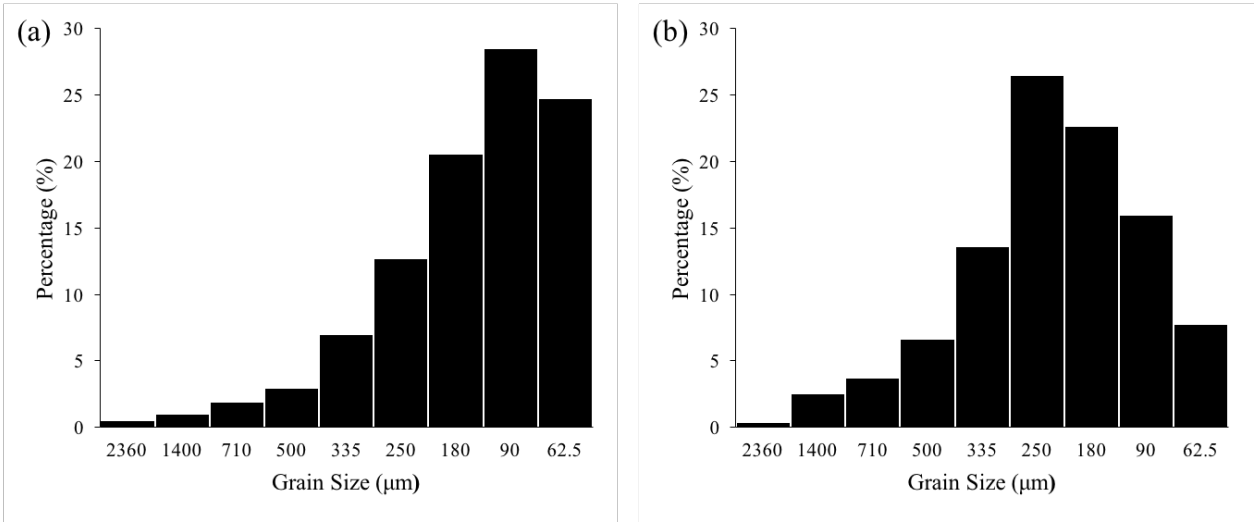
Table 2.4.1. The maximum and minimum measurements of saline at both sampling sites during early and end of emersion periods.

Sites	*Date*	*Emersion periods*	*Salinity*
Penna Beach	1st April 2014	1	36.6
	18th April 2014	2	36.6
	19th August 2014	1	29.8
	19th August 2014	2	30.3
Kings Beach	1st March 2014	1	34.9

20th October 2014	2	37.2
17th August 2014	1	24.4
17th August 2014	2	22.6

553

554 The grain size analyses from both sampling sites showed that the sediment composition at
555 Penna Beach is sandy mud, with ≥ 70% of sediments comprised of sediments with grain size
556 ≤ 180 μm, whilst that of Kings Beach is fine sand, with ≥ 70% of sediments made up of
557 sediments with grain size ≤ 250 μm (Fig. 2.4.1).



558

559 Figure 2.4.1. Sediment composition of grain size in proportion to each grain size category at
560 (a) Penna Beach and (b) Kings Beach.

561 The average concentrations of NO_x, DRP, and NH_4 were 22.75 ± 4.665 μg/L, 10.667 ± 0.972
562 μg/L, and 10.25 ± 1.169 μg/L, respectively (Fig. 2.4.2).

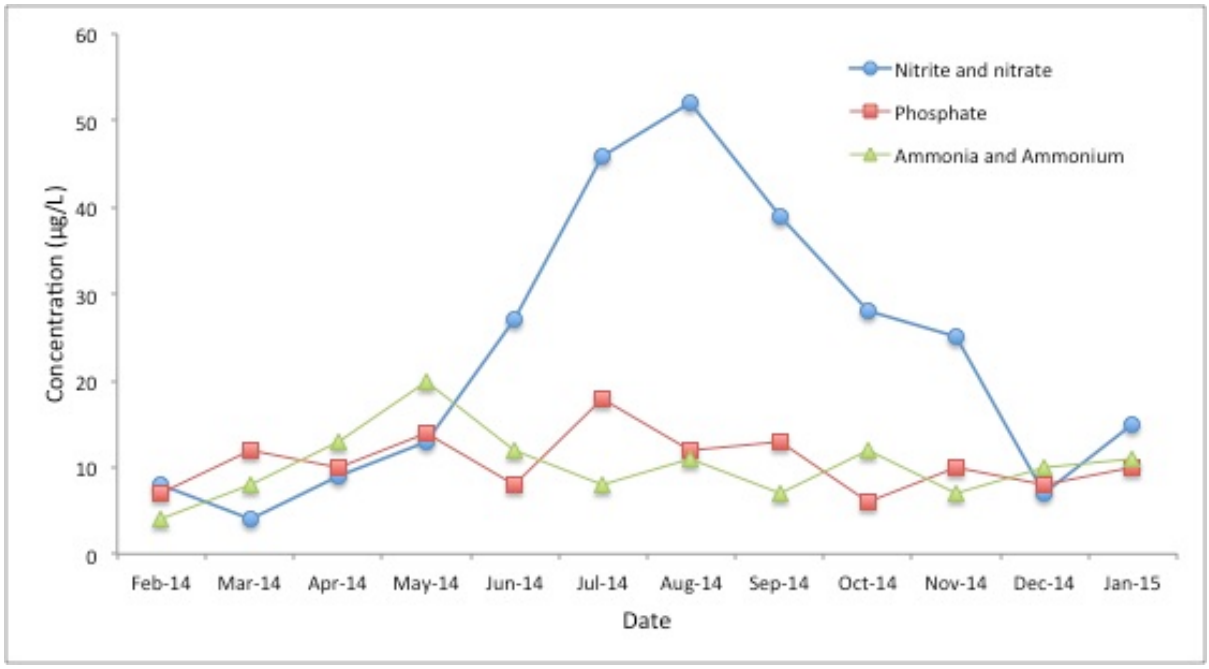
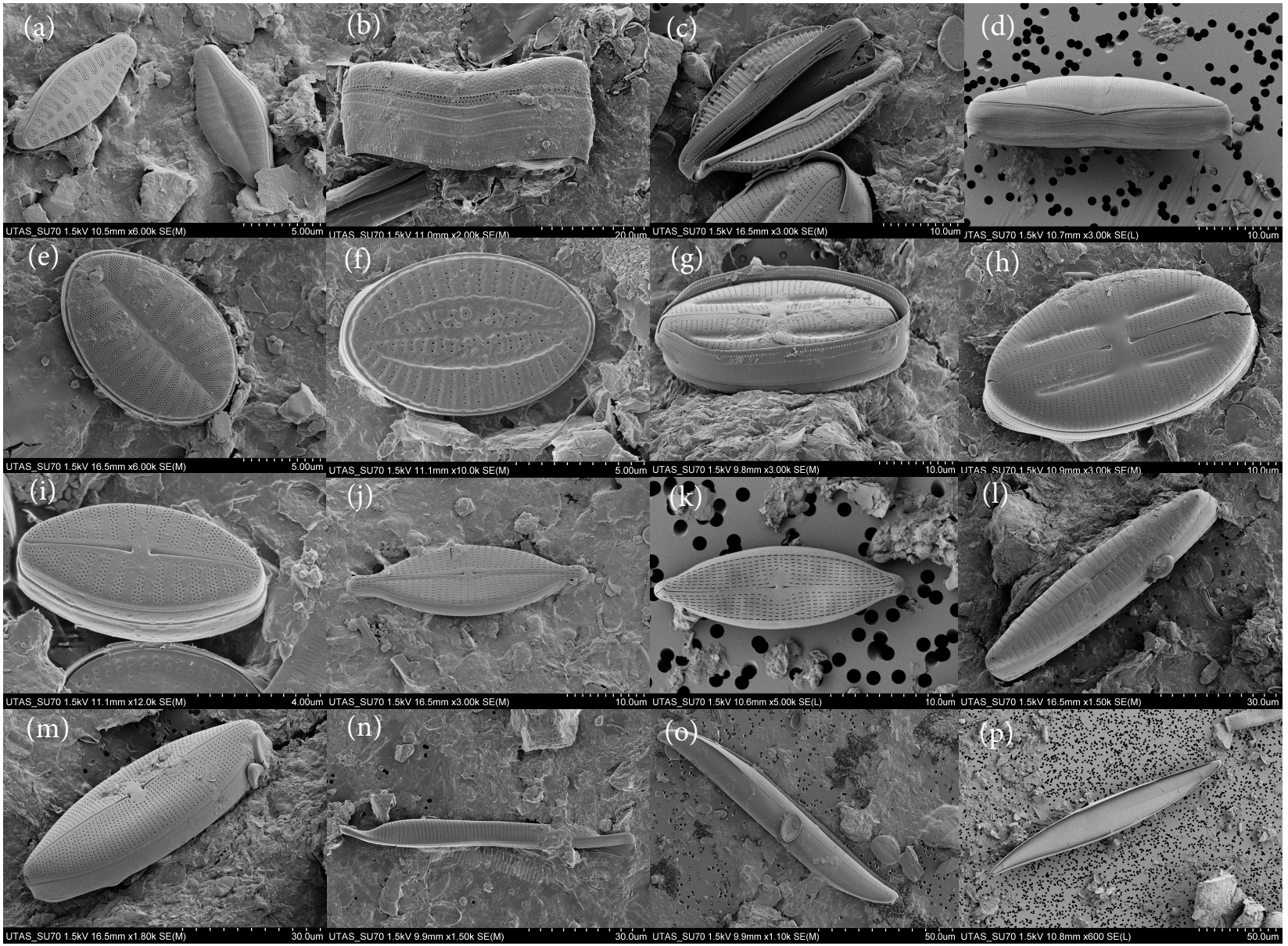


Figure 2.4.2. The annual fluctuation in NOx, DRP, and NH4 at the Derwent Estuary from February 2014 to January 2015. Data obtained from Coughanowr *et al.* (2015).

2.4.2. Species composition and diversity

At Penna Beach, the microphytobenthic communities were mainly dominated by small diatoms such as *Achnanthes brevipes*, *Cocconeis peltoides*, *Navicula jeffreyae*, and *Amphora laevissima*. On the other hand, Kings Beach was dominated by more diverse communities, with different sizes of diatoms such as *A. laevissima*, *Cymbella sumatrensis*, *Cocconeis costata*, *C. peltoides*, *Fallacia nyella*, *Achnanthes brevipes*, *N. jeffreyae*, *Diploneis weissflogii*, *Nitzschia commutata*, *Gyrosigma balticum*, and *Pleurosigma salinarum* (Fig. 2.4.3). Kings Beach has a more diverse MPB community, as it is equally dominated by MPB of different cell sizes, whilst the MPB community at Penna Beach is more dominated by smaller cell size microalgae, where MPB with cell size 10 µm made up almost half of the total abundance (Fig. 2.4.4).


(a)
UTAS_SU70 1.5kV 10.5mm x6.00k SE(M) 5.00um
(b)
UTAS_SU70 1.5kV 11.0mm x2.00k SE(M) 20.0um
(c)
UTAS_SU70 1.5kV 16.5mm x3.00k SE(M) 10.0um
(d)
UTAS_SU70 1.5kV 10.7mm x3.00k SE(L) 10.0um
(e)
UTAS_SU70 1.5kV 16.5mm x6.00k SE(M) 5.00um
(f)
UTAS_SU70 1.5kV 11.1mm x10.0k SE(M) 5.00um
(g)
UTAS_SU70 1.5kV 9.8mm x3.00k SE(M) 10.0um
(h)
UTAS_SU70 1.5kV 10.9mm x3.00k SE(M) 10.0um
(i)
UTAS_SU70 1.5kV 11.1mm x12.0k SE(M) 4.00um
(j)
UTAS_SU70 1.5kV 16.5mm x3.00k SE(M) 10.0um
(k)
UTAS_SU70 1.5kV 10.6mm x5.00k SE(L) 10.0um
(l)
UTAS_SU70 1.5kV 16.5mm x1.50k SE(M) 30.0um
(m)
UTAS_SU70 1.5kV 16.5mm x1.80k SE(M) 30.0um
(n)
UTAS_SU70 1.5kV 9.9mm x1.50k SE(M) 30.0um
(o)
UTAS_SU70 1.5kV 9.9mm x1.10k SE(M) 50.0um
(p)
UTAS_SU70 1.5kV 10.8mm x600 SE(L) 50.0um

576

Figure 2.4.3. Field Emission Scanning Electron Microscopy images of the dominant MPB species at both sampling sites, with *Achnanthes reidensis* (a), *Achnanthes brevipes* (b), *Amphora coffeaeformis* (c), *Amphora laevissima* (d), *Cocconeis costata* (e), *Cocconeis peltoides* (f), *Lyrella david-mannii* (g), *Fallacia nyella* (h), *Navicula jeffreyae* (i), *Navicula rhynchocephala* (j), *Navicula salinarum* (k), *Navicula normaloides* (l), *Navicula menisculus* (m), *Nitzschia commutata* (n), *Gyrosigma distortum* (o), and *Gyrosigma turgida* (p).

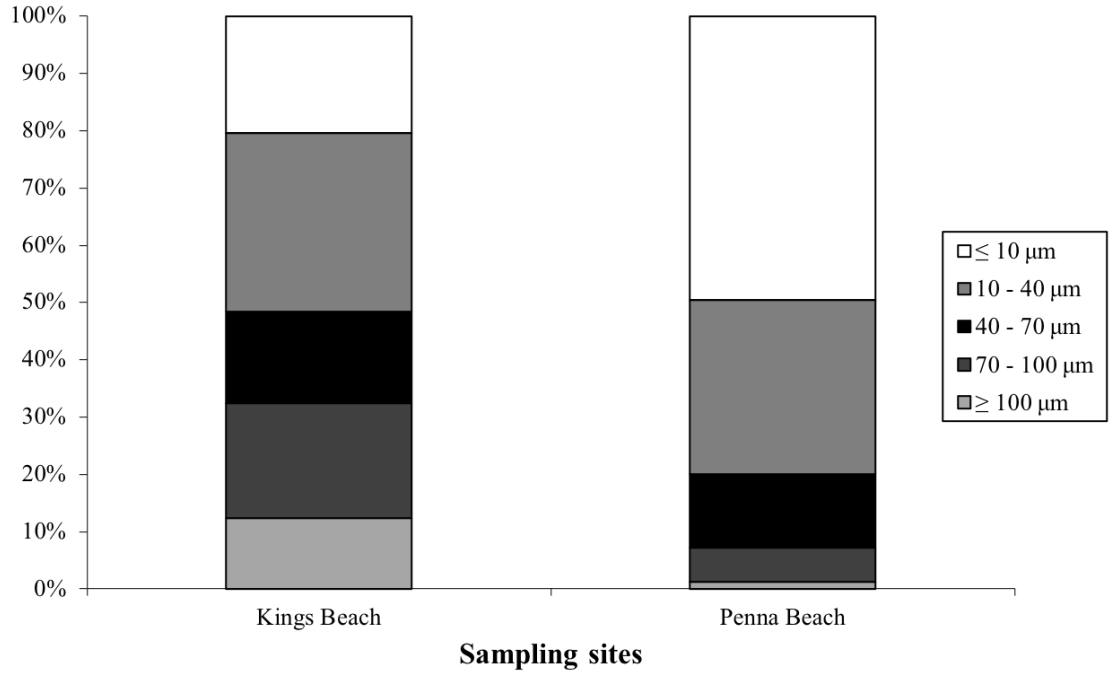


Figure 2.4.4. The percentage contribution of differently sized MPB species at (a) Penna Beach and (b) Kings Beach based on a monthly 200-cells count.

2.4.3. Chl.*a* concentrations

In this study, the chl.*a* concentration is considered as a proxy for microphytobenthic biomass. It differed significantly between sampling sites (ANOVA: p-value= 2 x 10^{-16}, F= 165.254, df= 1). There were no significant differences between seasons, and between the early and late emersion periods, as the maximum biomass in Penna Beach were 442.051 ± 22.409 µg m^{-2} during early emersion period on 6th June 2014, and 434.802 ± 31.998 µg m^{-2} during late emersion period on 1st April 2014. Kings Beach had a much lower biomass compared to that of Penna Beach, where its maximum biomass was 213.544 ± 14.731 µg m^{-2} during the early emersion period on 17th August 2014, and 242.739 ± 14.860 µg m^{-2} during the second emersion period on 23rd February 2014 (Fig. 2.4.5). Although significant differences in MPB biomass were observed between sites, ANCOVA results did not show any significant correlations between the MPB biomass and any of the environmental variables.

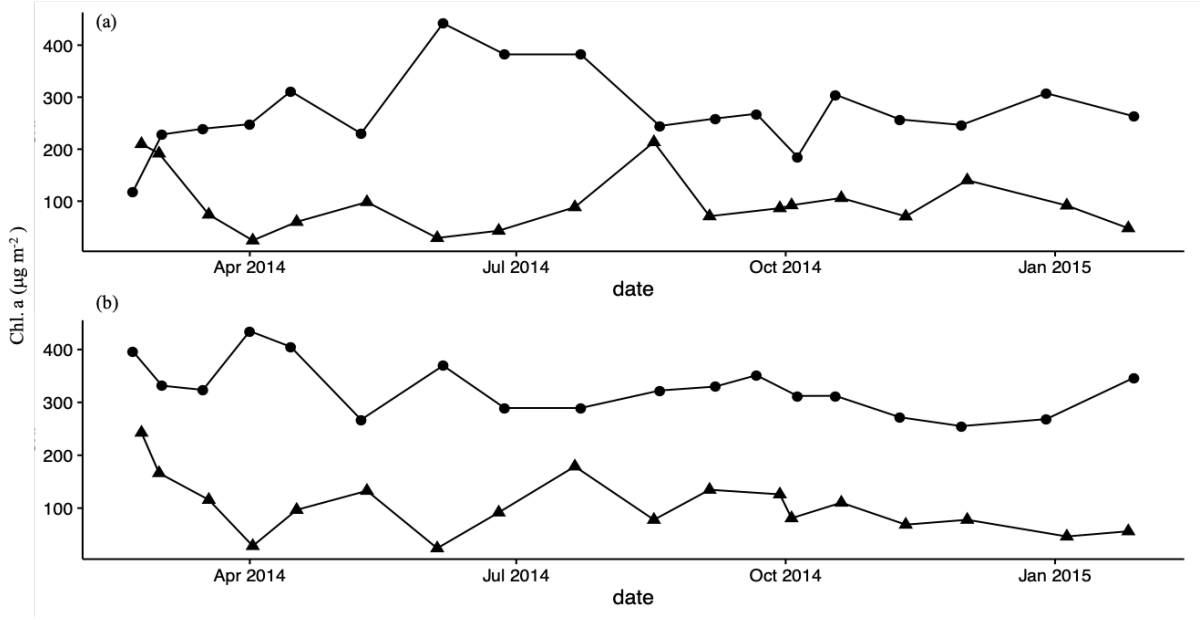
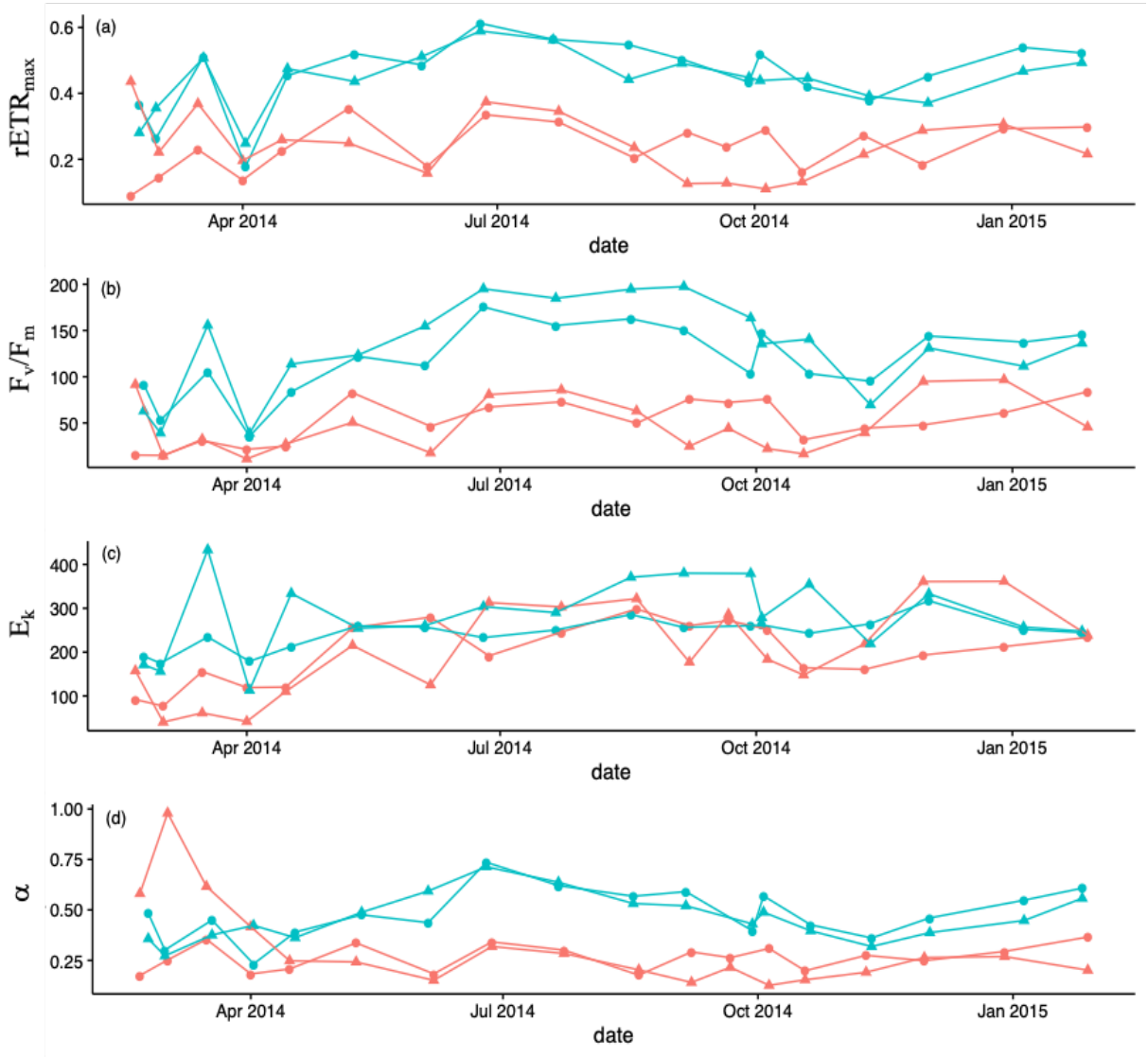


Figure 2.4.5. The seasonal microphytobenthic chl.*a* concentrations at Penna Beach (●) and Kings Beach (▲), at the beginning of the emersion period (a) and the late emersion period (b).

2.4.4. Chlorophyll fluorescence

RLCs were measured at both sampling sites independently. At Penna Beach, $rETR_{max}$ and E_k varied significantly between seasons (ANOVA: p= 0.0346, F= 3.304, df= 3 and ANOVA: p= 0.000862, F= 7.376, df= 3, respectively), where the values was higher during spring and summer. However, no significant differences between seasons were measured in F_v/F_m and α.

606 Furthermore, there were no significant differences observed between the early and late
607 emersion periods for all photosynthetic parameters. At Kings Beach, only $rETR_{max}$ varied
608 significantly between seasons (ANOVA: p= 0.000857, F= 7.384, df= 3), where elevated
609 $rETR_{max}$ were measured during winter.



610

611 Figure 2.4.6. The annual microphytobenthic photosynthetic performances at Kings Beach (blue
612 lines) and Penna Beach (red lines), during early (●) and late (▲) emersion periods, for F_v/F_m (a),
613 $rETR_{max}$ (b), E_k (c), and α (d).

Table 2.4.2. Analysis of Covariance on the relationship of MPB F_v/F_m and $rETR_{max}$, to different environmental variables. Significant p-values are in **bold**.

Sites	Components	Photosynthetic Parameters			
		Fv/Fm	*rETRmax*	α	*Ek*
Penna Beach	Light	0.115	**0.0365**	0.836	**0.242**
	Temp.	**0.0364**	**0.0169**	0.992	0.155
	Sal.	0.0600	**0.0287**	0.763	0.373
	Precip.	0.425	0.785	0.876	0.996
	Light: Temp	0.0745	**0.0267**	0.962	0.141
	Light: Sal.	0.108	**0.0362**	0.832	0.254
	Temp.: Sal.	**0.0331**	**0.0147**	0.991	0.147
	Light: Precip.	0.207	0.397	0.728	0.792
	Temp.: Precip.	0.315	0.521	0.812	0.884
	Sal.: Precip.	0.460	0.838	0.905	0.997
Kings Beach	Light	0.741	0.559	0.143	0.902
	Temp.	0.749	0.859	0.687	0.879
	Sal.	0.726	0.668	0.179	0.814
	Precip.	**0.0386**	0.0725	0.0525	0.277
	Light: Temp	0.734	0.651	0.352	0.949
	Light: Sal.	0.769	0.550	0.130	0.889
	Temp.: Sal.	0.760	0.821	0.550	0.841
	Light: Precip.	0.221	0.274	**0.368**	0.477
	Temp.: Precip.	**0.020**	**0.0406**	**0.0165**	0.235
	Sal.: Precip.	**0.0339**	0.0632	**0.0443**	0.260

ANCOVA results showed that irradiance, sediment temperature, precipitation, and salinity have significant effects on the photosynthetic performance of MPB, particularly F_v/F_m and $rETR_{max}$ (Table 2.4.2). Additionally, these environmental factors also showed significant combined effects on F_v/F_m and $rETR_{max}$. However, these correlations were not observed in E_k and α (Table 2.4.2).

Table 2.4.3. Analysis of Covariance for different environmental variables and MPB F_v/F_m and $rETR_{max}$. Significant p-values are in **bold**.

Environmental components	Extracellular carbohydrate composition		
	TCHO	MCHO	PCHO
Precipitation	**0.0101**	**0.00612**	0.718
Salinity: Precipitation	**0.0116**	**0.00665**	0.700

2.4.5. Composition of chl. *a*- normalised extracellular carbohydrate

As the extracellular carbohydrates correlated with the chlorophyll concentration measured in this study, they were normalised for chlorophyll in order to obtain the chl.*a*-normalised TCHO, MCHO, and PCHO. Chl.*a*-normalised TCHO concentrations showed significant variation between sampling sites (ANOVA: $p= 2 \times 10^{-16}$, F= 439.833, df= 1). Chl.*a*-normalised TCHO was much lower in Kings Beach compared to Penna Beach. The concentrations in Kings Beach ranged from 0.226 ± 0.004 (mg C [mg chl.*a*]$^{-1}$) to 3.361 ± 0.333 (mg C [mg chl.*a*]$^{-1}$) during early emersion and ranged from 0.195 ± 0.0138 (mg C [mg chl.*a*]$^{-1}$) to 1.247 ± 0.104 (mg C [mg chl.*a*]$^{-1}$) during the late emersion period. At Penna Beach, the chl.*a*-normalised TCHO ranged from 4.227 ± 0.150 (mg C [mg chl.*a*]$^{-1}$) to 10.702 ± 0.419 (mg C [mg chl.*a*]$^{-1}$) during the early emersion and from 3.147 ± 0.141 (mg C [mg chl.*a*]$^{-1}$) to 5.940 ± 0.194 (mg C [mg chl.*a*]$^{-1}$) during the late emersion period. No significant differences were measured between the early and late emersion periods at both sampling sites. Chl.*a*-normalised MCHO showed significant differences between sampling sites (ANOVA: $p= 2 \times 10^{-16}$, F= 345.668, df= 1). At Kings Beach, chl.*a*-normalised MCHO ranged from 0.136 ± 0.00541 (mg C [mg chl.*a*]$^{-1}$) to 2.762 ± 0.497 (mg C [mg chl.*a*]$^{-1}$) in the early emersion period and ranged from 0.102 ± 0.00779 (mg C [mg chl.*a*]$^{-1}$) to 0.829 ± 0.0774 (mg C [mg chl.*a*]$^{-1}$) in the late emersion period. At Penna Beach, chl.*a*-normalised MCHO ranged from 2.566 ± 0.179 (mg C [mg chl.*a*]$^{-1}$) to 6.694 ± 0.0884 (mg C [mg chl.*a*]$^{-1}$) during the early immersion period and ranged from 1.723 ± 0.0458 (mg C [mg chl.*a*]$^{-1}$) to 4.436 ± 0.0579 (mg C [mg chl. *a*]$^{-1}$) during the late emersion period. Similar to chl.*a*- normalised TCHO and MCHO, chl.*a*- normalised PCHO showed a significant difference between the sampling sites (ANOVA: $p= 2 \times 10^{-16}$, F= 452.712, df= 1).

Chl.*a*-normalised PCHO at Kings Beach ranged from 0.0686 ± 0.00316 (mg C [mg chl.*a*]$^{-1}$) to 0.599 ± 0.164 (mg C [mg chl.*a*]$^{-1}$) during the first emersion period and ranged from 1.057 ± 0.074 (mg C [mg chl.*a*]$^{-1}$) to 0.0573 ± 0.0112 (mg C [mg chl.*a*]$^{-1}$) during the late emersion period. At Penna Beach, the chl.*a*-normalised PCHO ranged from 0.897 ± 0.150 (mg C [mg chl.*a*]$^{-1}$) to 4.008 ± 0.344 (mg C [mg chl.*a*]$^{-1}$) during the first emersion period and ranged from 1.261 ± 0.116 (mg C [mg chl.*a*]$^{-1}$) to 2.203 ± 0.161 (mg C [mg chl.*a*]$^{-1}$) during the late emersion period. Similar to chl.*a*-normalised TCHO, both chl.*a*- normalised MCHO and chl.*a*-normalised PCHO did not show any significant differences between early and late emersion periods. All chl.*a*- normalised carbohydrates only varied significantly between sampling sites but did not show any significant variation between seasons, and sampling periods either.

2.5. Discussion

This study investigated the photosynthesis and extracellular carbohydrate production of MPB communities at two Tasmanian beaches in response to their environment during the early and at the end of emersion periods throughout a year (between 2014 and 2015)

The MPB biomass varied significantly between Penna Beach and Kings Beach, but it was not affected by the environmental factors, as there were no strong correlations between MPB biomass and the environmental parameters. This observation is consistent with results from some previous studies (Lee and McMinn, 2013). Working on MPB in Ria Formosa (Portugal) from 2006 to 2008, Brito *et al.* (2009) reported that MPB chlorophyll did not correlate with tidal range, wind speed, solar irradiance, water temperature, salinity and nutrient concentrations. Similar results were also reported from the Nakdong River estuary (Korea), where MPB biomass was not significantly correlated to pore water nutrients, light intensity and salinity . Furthermore, MPB biomass from Ria Formosa study did not vary seasonally, even though the environmental variables changed significantly . However, on the other hand, these authors observed higher MPB biomass in spring, when irradiance and sediment temperature were at optimum conditions for photosynthesis. The Ria Formosa MPB biomass was also found to be highly correlated with light intensity, which usually occurred during spring and summer (Brito *et al.*, 2009).

Nutrient levels are one of the crucial factors in controlling MPB biomass. In this study benthic chl. *a* was found to be affected by nutrients, especially by ammonium and silicate rather than by nitrate and phosphate . Both ammonium and silicate were unlikely to be a limiting as their concentrations at both sites remained relatively high, and well above concentrations considered as limiting (Cook et al., 2004; 2007) (Fig. 2.4.2). Nutrient levels at both sites investigated in this study remained relatively high during all seasons, as nutrients of coastal areas are being repeatedly replenished through tidal activity.

Short-term variability holds important contribution in the microphytobenthic community as well, where vertical migration may take over and affect the biomass of MPB in the sediment (Serôdio et al., 2001, Perkins et al., 2010). MPB at Penna Beach of this study showed significant variation in the biomass before and after the low tide period, where the biomass was relatively higher during the late emersion period, showing that more MPB were at the sediment surface, mainly to acquire irradiance for photosynthesis. Several studies have demonstrated the

importance of short-term variability in the biomass of intertidal microphytobenthos, where migratory rhythms become important and mask the significance of photophysiological adaptation (Serôdio et al., 2001; Mitbavkar and Anil, 2004; Jordan et al., 2008; Du et al., 2010; Perkins et al., 2010). This changes in biomass was not observed at Kings Beach, which may be due to the deeper light attenuation into the coarser sediments. Unfortunately, the light attenuation was not measured in this study, hence assumption can only be made that the MPB did not migrate to the sediment surface for photosynthesis. Serôdio et al. (2001) have also shown a predominance of fortnightly over seasonal variability in the MPB productivity for the Tagus Estuary (Portugal), where tidal amplitude that changes at short time periods has a greater impact on the migratory patterns of MPB. They report that MPB vertical migration was often dependant on the occurrence of neap and spring tide events, which happen fortnightly. Additionally, changes in biomass at Penna Beach during summer and spring were similar to these observations from Portugal (Serôdio et al., 2001). In contrast there were no differences during winter, when overall MPB biomass was relatively low. This pattern can be explained by vertical micro-migration of the epipelic diatoms, commencing at the beginning of the emersion period, with cells slowly migrating away from excessive irradiance during spring and summer. Lee and McMinn (2013) also found that in a study of MPB in Tasmania during winter, freezing temperatures were found to have on little effect on MPB physiology. As a result, during winter when light availability is minimal (as shown in the present study) MPB may have stayed at the surface most of the time, in order to obtain maximum irradiance for photosynthesis.

Sediment grain size is also another important factor in affecting MPB biomass (Jesus et al., 2006, Maggi *et al.*, 2013). MPB biomass in this study varied significantly between sampling sites, where Penna Beach had higher biomass than that of Kings Beach. Serôdio et al. (2001) have shown that MPB biomass was highly stratified with depth in the surface sediments of the mudflats, while sandy areas had a more homogeneous distribution with sediment depth (down to 3 mm). Penna Beach and Kings Beach comprised of fine sediments and coarser sediments, respectively; hence higher microphytobenthic biomass was observed at Penna Beach than that of Kings Beach. Similarly, negative relationships between the percentage of fine sediments and MPB biomass have been observed in the Neuse River estuary (North Carolina), where the highest sedimentary chl.*a* levels generally occurred in sediments with a lower proportion of fine particles (diameter ≤ 125 μm), with chl.*a* being found down to 3.5 cm deep in the sediment (Cahon, 1999). The main reason for these observations is likely due to the deeper light penetration and/or increased physical mixing in the sandy sediments, leading to a more

721 homogeneous distribution of MPB biomass into deeper sediment layers. Hence, higher MPB
722 biomass at the sediment surface of finer sediment does not mean that the total MPB biomass is
723 relatively higher in finer sediment sites compared to sandy sites, where a more homogeneous
724 stratification in MPB biomass can result in an increased total biomass.

725 Microphytobenthic photosynthetic performance in the present study showed obvious short-
726 term variations at both Penna Beach and Kings Beach At both sites MPB photosynthetic
727 parameters were dependant on environmental drivers. Irradiance, sediment temperature,
728 salinity and precipitation were all major factors responsible for seasonal changes in MPB
729 photosynthesis in this study, in particularly their combined effects. In natural ecosystems, MPB
730 can be readily affected by irradiance and temperature during low tide. In a laboratory study,
731 Morris and Kromkamp (2003) reported that high temperature and irradiance caused a major
732 decrease in F_v/F_m when thermal stress was only apparent when the temperature exceeded 25 °C.
733 They also reported that the optimum growth temperatures for the temperate benthic microalga,
734 *Cocconeis sublittoralis* in culture was within the range of 18 °C to 25 °C. In this study the MPB
735 at both sites showed higher F_v/F_m and $rETR_{max}$ during winter when the irradiance and
736 temperature were lower. These results are similar to those of Jordan et al. (2008), who found
737 that F_v/F_m values were higher during winter at Browns River, Tasmania. In temperate regions,
738 intertidal biofilms can be exposed to high temperatures and irradiances exceeding 2000 μmol
739 photon m^{-2} s^{-1} (Cartaxana *et la.*, 2016). In addition to light and sediment temperature, rapid
740 increases in salinity during late spring or summer can also impose significant stress (Lee and
741 McMinn, 2013). In the present study salinity alone did not have a significant effect on MPB
742 photosynthetic performance, but a combination of salinity with other environmental factors
743 reduced F_v/F_m and $rETR_{max}$ values. High salinity events can also occur in all seasons,
744 particularly when wind speed is high, driving desiccation on the sediment surface. Serôdio et
745 al., (2009) showed that there was significant decrease in $rETR_{max}$ microphytobenthic
746 communities when they were subjected to high salinity and irradiance. Photosynthetic activity
747 of benthic diatoms is highly species-specific, which may be the cause of the difference in the
748 measured photosynthesis of current study. Kings Beach had higher population of bigger size
749 MPB than that of Penna Beach, which may have resulted in the difference of photosynthesis
750 between these sites. Yun et al. (2010) supported this observation, where bigger cells, that
751 migrate more slowly, with smaller surface area to volume (SA/V) ratios, showed higher F_v/F_m
752 than smaller cells with bigger ratios. Similar trends were also observed by Serôdio et al. (2001)
753 in the Tagus Estuary (Portugal). This observation is consistent with the F_v/F_m values measured

754 in this study, where those from Kings Beach with a higher biomass with bigger cell size (i.e.
755 smaller SA/V ratio) had a higher photosynthetic performance than those from Penna Beach,
756 which had a greater abundance of smaller cells. Different dominant species also have different
757 nutrient uptake rates that may additionally alter F_v/F_m . The higher E_k, and α values observed
758 at Kings Beach also showed that the MPB at Kings Beach was acclimated to higher irradiance.
759 Yun et al. (2010) further showed that bigger MPB species have greater tolerance to higher
760 temperatures and irradiances, and thus have a better photosynthetic performance than smaller
761 microphytobenthic species. This explains why the MPB in Kings Beach are more likely to
762 undertake photoacclimation to photo-regulate in response to higher light intensity.

763 Photosynthetic overflow metabolism is a well-established mechanism in MPB communities
764 that occurs during their growth (Marañón et al., 2004; Lundkvist et al., 2007; Oakes et al.,
765 2010). Oakes et al. (2010) further found that almost 90% of glucose was produced and excreted
766 as extracellular carbohydrates during emersion period. Polysaccharides produced by MPB are
767 highly variable in size and these polymers can be rapidly hydrolysed into smaller molecules,
768 preferably glucose. This may explain the higher concentration of chl.*a*- normalised MCHO
769 observed in the present study, where it is often greater than that of chl.*a*- normalised PCHO. In
770 their experimental study Lundkvist et al. (2007) found that overflow metabolism occurred at
771 low temperatures, as MPB did not grow as rapidly as they were under higher temperatures,
772 thus the photosynthetically fixed C was transferred to EPS, especially to colloidal
773 carbohydrates. This phenomenon was not observed in this study, as extracellular carbohydrate
774 concentrations remained constant, even during winter. The results from this study suggest that
775 the rate of overflow production by MPB is not significantly affected by the temperature.

776 MPB biomass showed significant differences between sites and between early and late
777 emersion periods but no differences with season. This can potentially be explained by changing
778 grazing pressure. Although not specifically studied here, MPB supports diverse benthic food
779 webs and is consumed by the macrobenthos. MPB biomass in this study was highest in summer,
780 lower in late autumn and lowest in winter. The reduction in grazing pressure during autumn
781 and winter might have in turn allowed the MPB biomass to increase. As the major component
782 of extracellular carbohydrates produced during the ermersion period was glucose, which
783 represented about 90% of the secreted carbohydrate, it was probably also taken up by bacteria.
784 McKew et al. (2013) have also shown that MPB cultures collected and isolated from Colne

Estuary (United Kingdom) showed that the microphytobenthic EPS degradation was highly correlated with the presence of bacterial communities.

2.6. Conclusion

This study highlighted that the relationships between microphytobenthic photosyntheisis, and extracellular carbohydrate production, and different environmental variables are not simple, as they are also likely to be influenced by a complex set of interacting factors (Cahoon *et al.*, 1999). The present study showed that MPB in the Tasmanian waters are well adapted to their dynamic environment by performing physiological and behavioural responses, i.e. photoacclimation and migration, respectively, in response to environmental variables. Consequently, changes in their surroundings do not often drive them into 'stressed' conditions. Significant variations in photosynthetic parameters, microphytobenthic biomass, and extracellular carbohydrate production between site are most likely due to the sediment grain size difference between the sampling locations. In addition to sediment grain size, other environmental factors such as irradiance, sediment temperature, salinity, and nutrient levels were also important in affecting the performance of MPB in the tidal flats. For future investigations, sampling of different sediment depth profiles of MPB performance, an overall higher sampling frequency, and more sophisticated carbohydrate analyses, such as introducing the biosensors to measure the real-time concentration changes in the targeted sugars are recommended.

Chapter 3: Determining the performance of tropical microphytobenthic assemblages in response to various combined effects of different environmental stressors

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3.1. Abstract

Microphytobenthos (MPB) are unicellular microorganisms mainly inhabiting in the photic zone of tidal flats. MPB communities are generally dominated by diatoms, with additional small proportions of dinoflagellates and euglenoids. MPB exert a significant role in contributing to the coastal total primary production.

Several sampling sites at North West coast of the peninsular Malaysia were chosen to study the microphytobenthic photosynthesis and extracellular carbohydrate production in response to different variables in the field. Species composition and abundance were measured in order to investigate the variation among sampling sites. Sediment samples were collected to measure the microphytobenthic chl.*a* concentration, extracellular carbohydrate concentrations, as well as the photosynthetic performance i.e. F_v/F_m and $rETR_{max}$. Sediment samples collected for extracellular carbohydrate concentrations were extracted and hydrolysed into 3 fractions: total organic carbohydrate (TCHO), monosaccharides (MCHO), and polysaccharide (PCHO).

Chl.*a*, TCHO, MCHO, and PCHO varied between sampling sites. Highest and lowest chl.*a* concentrations were measured at Teluk Bahang and Tanjung Rhu T3, averaging 18.390 ± 3.413 and 2.389 ± 0.390 μmol L^{-1}, respectively. Highest TCHO was observed at Tanjung Rhu T3, averaging at 53.572 ± 11.380 μmol L^{-1}, while lowest TCHO was observed at Teluk Air Tawar, averaging at 5.524 ± 0.856 μmol L^{-1}. The chl.*a*, TCHO, MCHO, and PCHO were mainly affected by sediment temperature, salinity, and irradiance. Nitrate was the only nutrient having a significant impact on the chl.*a* concentration, and all nutrient levels including nitrate, ammonia, and phosphate did not have significant effects on the TCHO, MCHO, and PCHO. PCHO concentrations were high at all sampling sites, comprising more than 60% of TCHO at each site. MCHO: PCHO ratios further showed that the ratios were almost 1:3 at all sampling sites. Measured chlorophyll fluorescence showed that the MPB at all sampling sites were not experiencing optimal conditions with relatively low photosynthetic efficiency (F_v/F_m) values at all sampling sites. Maximum F_v/F_m and $rETR_{max}$ were measured at Teluk Bahang, averaging at 0.360 ± 0.073 and 91.339 ± 4.756, in the top 6 mm sediment, respectively. Meanwhile, lowest F_v/F_m values were measured at Teluk Air Tawar, averaging 0.180 ± 0.019, while lowest $rETR_{max}$ values were measured at Gertak Sanggul, averaging at 29.367 ± 4.268 at the surface 6 mm of the sediment. Highest and lowest E_k values were measured at Tanjung Rhu 3 and Gertak Sanggul, averaging at 399.914 ± 50.188 μmol photons m^{-2} s^{-1} and 109.988 ± 19.947 μmol photons m^{-2} s^{-1}, respectively. Highest and lowest α values were measured at Teluk

Bahang and Jerejak, averaging at 0.344 ± 0.089 and 0.154 ± 0.0133, respectively, at the surface 6 mm of the sediment. Unlike chl.*a* and extracellular carbohydrate, the microphytobenthic photosynthetic performance in the current study did not seem to be affected by sediment temperature, salinity, and irradiance. The photosynthesis was more being affected by nutrient levels, where nitrate and ammonia exerted distinctive impacts on F_v/F_m and α., but nutrients did not have significant impact on $rETR_{max}$ and E_k.

The site-specific chl.*a* concentrations were mainly controlled by the sediment composition of the sampling sites, which were categorised as muddy, sandy mud, and sandy sites, respectively. The insignificant variation between sampling depths at each site was mainly due to the suspected high resuspension that occurred during the sampling periods, which was during the rainy period of dry season in Malaysia. As a result, the microphytobenthic community was more homogenised throughout the sampled depths. Additionally, as sampling was undertaken during the monsoon season, irradiance appeared not to drive the MPB to respond either behaviourally or physiologically to this environmental factor. This is because the irradiances were not extreme enough to inhibit the MPB performance, due to the extensive cloud cover that occurred during the monsoon season (less than 200 μmol photon m^{-2} s^{-1}). PCHO concentrations were relatively higher than MCHO at all sampling sites, possibly as a result of organic matter supplied by river run-off. Additionally, previous studies have shown that there was lower MPB bacterial abundance during the monsoon season, which will reduce the PCHO degradation by bacterial consumption. Furthermore, although MPB produce MCHO in response to extreme environment as an overflow, environmental conditions from the current study were not extreme enough to drive the MPB into a stressed condition. Hence, MCHO concentrations were relatively lower compared to PCHO.

3.2. Introduction

The photic zones of intertidal areas make a significant contribution to coastal ecosystem production, due to their high primary productivity. This intertidal primary production is largely contributed by microphytobenthos (MPB), accounting for up to 50% of the total primary production in coastal areas (Underwood et al., 2005). MPB communities are mostly dominated by diatoms, although other groups of phototrophs such as cyanobacteria and euglenoids are also present. The diatoms can be grouped as epipelon (motile) and episammon, which exhibit migratory rhythms synchronised with diurnal and tidal cycles and attached to the sand grains, respectively. They are concentrated in the shallow water environments, where they are exposed to a number of environmental stressors typical for the intertidal zone.

Intertidal environments are highly dynamic systems and subject to extremes in environmental variability including irradiance (Serôdio et al., 2005; 2006; Du et al., 2010; Perkins et al., 2010; Cartaxana et al., 2013), temperature (Morris and Kromkamp, 2003; Defew et al., 2004; Hancke et al., 2008; Yun et al., 2010; Vieira et al., 2013; Lee and McMinn 2014), immersion (Miles and Sundback, 2000; Mitbavkar and Anil, 2004), and nutrient variations (Underwood, 2002). These environmental stresses impact MPB communities on multiple spatial and temporal scales. From time scales as little as an hour or a minute and spatial scales of less than a millimetre (i.e. sediment depth), to large-scale seasonality influencing entire coastal ecosystems. Tropical intertidal zones are considered to be highly sensitive to environmental change, due to the large impact that small changes can have on the entire ecosystem (Mitbavkar and Anil, 2006; Khodse et al., 2010).

Because of the environmental variability of their habitats, MPB communities are frequently experiencing these "stressed" environments (Facca and Sfriso, 2007; Lee and McMinn, 2013). As a result, these microphytobenthic diatoms have developed different physiological and behavioural mechanisms to adapt to changing environmental conditions, such as regulating their photosynthetic performance through processes such as the xanthophyll cycle (Jordan et al., 2010; Blanchard et al., 2004; Jesus et al., 2009; van Leeuwe et al., 2008). This is performed by manipulating the rate of the diadinoxanthin (DD) cycle, which involves a rapid and reversible conversion from DD (epoxide form) to diatoxanthin (DT) (de-epoxidated form) (Müller et al., 2001). In addition to these physiological mechanisms epipelic diatoms also use behavioural mechanisms, such as migration, to position themselves at the optimal depth within the sediment to prevent photo-damage (Jordan et al., 2010; Du et al., 2010; Jesus et al., 2009;

McLachlan et al., 2009). In addition to elevated irradiances, biofilms are also exposed to high salinity and desiccation, caused by evaporation or precipitation during tidal exposure. High light and temperature coupled with low nutrient concentrations, may lead to photoinhibition but this is rarely seen in temperate ecosystems (Kromkamp et al., 1998; Dodds et al., 1999; Underwood and Kromkamp, 1999). However, as tropical marine waters generally have very low concentrations of dissolved inorganic nutrients (Dizon and Yap, 1999, Le Borgne et al., 1997) and higher irradiances, it is possible that photoinhibition may be more common in low latitude MPB.

Extracellular polymeric substance (EPS) production is an important characteristic of MPB communities. Production of EPS, for instance, has been observed during the vertical migration of diatoms to aid their movement within the sediment (Underwood and Smith, 1998; Hoagland et al., 1993; de Brouwer and Stal, 2001). Extracellular carbohydrates are also produced as photosynthetic overflow under unfavourable conditions (Smith and Underwood, 2000; Alcoverro et al., 2000; Staats et al., 2000b; de Brouwer and Stal, 2002). Previous studies have shown that mucilage, consisting of low molecular weight carbohydrates, can also be produced as a response to high light and nutrient limitation. Under these conditions, excess carbon is released from the cells as dissolved organic carbon (DIC) (Smith and Underwood, 2000; Staats et al., 2000b), whereby excess photosynthetic products are actively released to the external medium when the C fixation rate greatly exceeds the rate of macromolecular synthesis. Under nutrient limited conditions fixed carbon is predominantly synthesized into carbon-rich storage products (i.e., carbohydrates, polysaccharides), as the lack of nutrients confines the synthesis of other cell components (Mueller et al., 2016; Konopka and Schnur, 1981; Hama and Yanagi, 2001). Photosynthetic overflow acts as a protective mechanism by MPB in response to nutrient limited conditions. Most previous studies have focused on temperate MPB communities (Brotas et al., 1995; Underwood and Kromkamp,1999; Thornton et al., 2002; Jordan et al., 2008; Jesus et al., 2009; Jordan et al., 2010; Lee and McMinn, 2013), and there have been only a few tropical studies (Mitbavkar and Anil, 2002; Underwood, 2002; Mitbavkar and Anil, 2004; Mitbavkar and Anil, 2006; Mitbavkar and Anil, 2008; Khodse et al., 2010; de Oliveira et al., 2011). Coastal ecosystems are increasingly threatened by human population growth and urbanisation, resulting in habitat loss and degradation due to coastal eutrophication and increased sedimentation (Tappin, 2002). The development of sustainable management strategies for such regions requires that the ecology of these habitats, and their response to environmental perturbations, is well understood (Crooks and Turner, 1999). Furthermore,

935 tropical marine ecosystems generally have very low nutrient concentrations (1-2 μM or less)
936 (Dizon and Yap, 1999; Le Borgne et al., 1997) and these low nutrient concentrations and higher
937 irradiances are likely to result in increased concentrations and production rates of DOC/EPS in
938 tropical biofilms compared with those found in temperate systems. This study focuses on the
939 production of extracellular carbohydrates by MPB in response to changing environmental
940 conditions during the monsoon season in Malaysia. The aims are (1) to investigate the
941 relationship between the extracellular carbohydrate production and the microphytobenthic
942 F_v/F_m and $rETR_{max}$, (2) to determine the major environmental variables that are affecting the
943 extracellular carbohydrate production during the raining period of the dry season.

3.3. Materials and Methods

3..3.1. Sampling sites

This study was undertaken at 7 sampling sites along the northwest coast of Peninsular Malaysia. The sites were located at Tanjung Rhu (6°27’04"N, 99°49’25"E) on Langkawi Island, Matang in Perak (4°49’14"N, 100°40’16"E), Teluk Bahang (5°27’44"N, 100°12’14"E), Gertak Sanggul (5°16’55"N, 100°11’51"E), Jerejak (5°20’42"N, 100°18’44"E), Gelugor on Penang Island (5°21’26"N, 100°18’56"E) and Teluk Air Tawar at Butterworth (42°53’44"N, 147°20’01"E) (Fig. 3.3.1). These sampling sites each have different sediment characteristics. Teluk Bahang and Matang were muddy sites, Jerejak, Gelugor and Teluk Air Tawar were sandy mud areas, and Gertak Sanggul was a sandy site. There were 3 sampling locations at Tanjung Rhu, and their sediment characteristics were either sandy mud or sandy; they were all submerged during low tide.

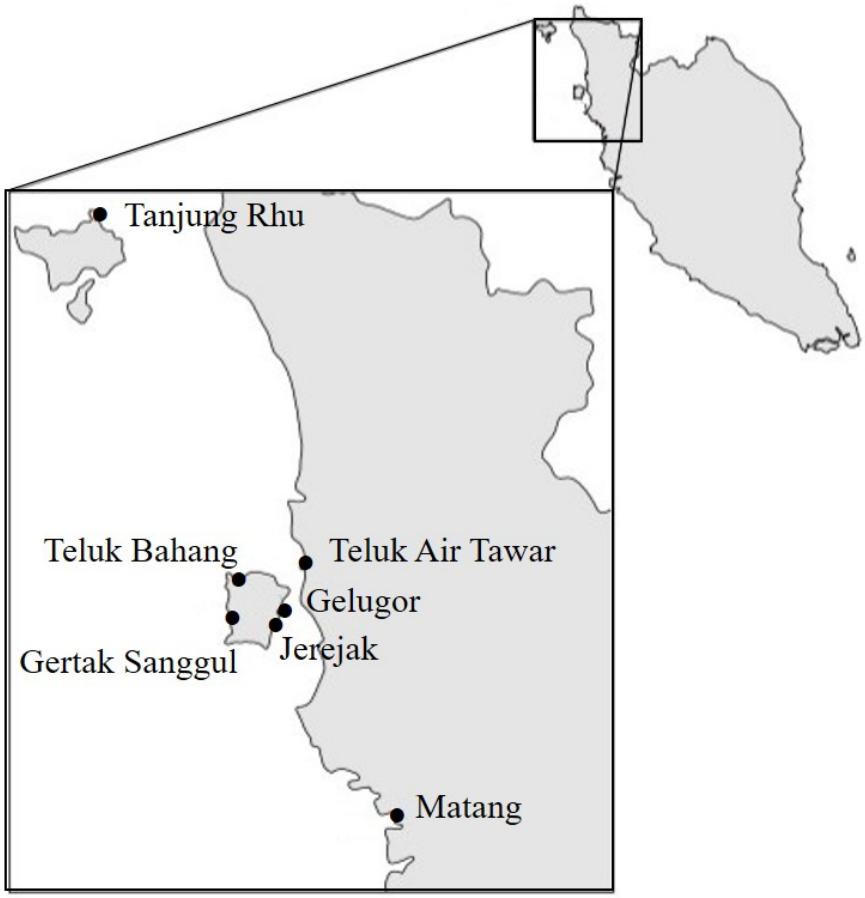

Figure 3.3.1. Sampling sites selected for this study along the west coast of Peninsular Malaysia.

3.3.2. Environmental data

The light intensities were measured using a Li- Cor Biosciences light meter (LI-250A, LI-COR, Nebraska, USA). Vertical temperature profiles from within the sediment were measured prior sampling by inserting a temperature probe (HI 766C) (K-type thermocouple thermometer, HI935005, Hanna Instruments, Woonsocket, Rhode Island, USA) vertically into the sediment at 1 mm intervals, down to 10 mm. Surface water salinity was obtained using an ATAGO E-Line refractometer (ATC Range, ATAGO CO. Ltd., Japan). Sediment grain size was analysed using the standard sieving method and was categorised according to Folk (1974).

Nutrient analysis was undertaken by collecting 250 ml water samples using a 250 ml polyethylene bottle. Samples were kept in a freezer at -20°C until further analysis. The concentration of nitrate (mg NO_3 –N/L), ammonia (mg NH_4 – N/L) and orthophosphate (mg PO_4^{2-} – N/L) were determined following the methods of Strickland et al. (1972). In brief, the pore water samples were filtered through a Whatman GF/C glass microfibre filter paper (47 mm in diameter, 1.2µm in pore size) to eliminate the suspended particles. The concentrations of each nutrient were measured by taking the absorbance readings of the samples using SHIMADZU UVmini-1240, UV-VIS spectrophotometer (Japan) against the plotted standard curves (Strickland et al., 1972).

3.3.3. Microphytobenthic species composition and abundance

Relative species identification at all sites was determined using Scanning Electron Microscopy (SEM). The top 5 cm sediment samples were collected from the sites and preserved in Lugol's iodine solution prior to preparation. Hydrogen peroxide (H_2O_2) was used to clean the preserved samples. The samples were filtered through 2 µm membrane filters and mounted on aluminium SEM stubs with double adhesive tape. The stubs were examined using a Hitachi SU-70 Analytical Field Emission Scanning Electron Microscope (FE-SEM) (Hitachi, Naka Division, Ibaraki Prefecture) and species identification was carried out following Saunders *et al.* (2010). MPB abundance was determined by counting 200 cells from fresh sediment samples using a Compound Light Microscope (Olympus, Notting Hill, Victoria) and the relative abundance was calculated.

3.3.4. Sampling protocols

Sample collection at each sampling site was carried out during low tide between May 2015 and July 2015. Sediment samples were collected by inserting a 45 mm diameter corer into the sediment. The collected samples were then sub-sectioned into 2 mm intervals down to 6 mm.

3.3.5. Microphytobenthic biomass

Three sediment samples for chl.*a* analysis were extracted in 10 mL of methanol in the dark at the field and were kept in an ice box with ice packs for at least 8 hours. The extractants were measured on a Turner Design 10AU fluorometer (Sunnyvale, CA, USA) using the acidification methods (Holm-Hansen et al., 1965). Chl.*a* concentrations were calculated by subtracting the acidified readings from the initial readings and multiplying by the acidification factor.

3.3.6. Chlorophyll fluorescence analysis

Photosynthetic performance was determined for each sample using a high-resolution Pulse Amplitude Modulated fluorometer (Water PAM; Waltz, Effeltrich, Germany), following Lee and McMinn (2013). Samples were mixed with 10 mL of filtered seawater and left in the dark for 30 minutes to dark-adapt. Rapid Light Curve (RLC) measurements were taken under software control (WinControl, Walz Gamb, Effliterch) via a light treatment consisting of a saturating pulse of light followed by eight consecutive 10 s intervals of increasing actinic light of 0, 105, 162, 242, 346, 484, 632, 980, and 1351 μmol photon $m^{-2}s^{-1}$ and the photosynthetic parameters were obtained following Ralph and Gademann(2005) (Table 3.3.1).

Parameters	Definition	Units
α	Initial slope of the relationship between PAR and rETR	Dimensionless
E_k	Light saturation parameter	μmol photons m^{-2} s^{-1}
F_v / F_m	Maximum photochemical efficiency	Dimensionless
$rETR_{max}$	Maximum relative electron transport rate	Dimensionless
PAR	Photosynthetic active radiation	μmol photons m^{-2} s^{-1}

Table 3.3.1. Parameters and definitions used in this study.

3.3.7. Extracellular carbohydrate analysis

Extracellular carbohydrate was extracted from the sediment samples following the protocol of de Brouwer and Stal (2001). In brief, sediment samples were initially extracted in MQ water at 30°C for an hour and then were kept at -20°C for further analysis. Extracted carbohydrates were measured spectrophotometrically based on protocols developed by Myklestad et al. (1997) and modified by Hung and Santschi (2001) to obtain the total extracellular carbohydrate (TCHO). In brief, 1 mL of extracted sample was mixed with 1 mL of Potassium Ferricyanide

and incubated in boiling water for 10 minutes. 1 mL of Ferric Chloride and 2 mL 2, 4, 6-Tripyridyl-s-Triazine (TPTZ) were then immediately added and mixed well on a Vortex-mixer. After 30 minutes, the relative absorbance of the samples at 595 nm was determined with a Cintral UV/Vis spectrophotometer (PerkinElmer, Waltham, Massachusetts, USA) against distilled water. The analysis was carried out in the dark or with low levels of red light, due to the high light sensitivity of the analytical reagents (van Oijen et al., 2003). Prior to analysis; all glassware was acid washed with 10% hydrochloric acid (HCl) for at least 2 hours and rinsed 3 times with MQ water. Concentrations of all extracellular carbohydrate fractions were then normalised against chl.*a* concentrations (μg C [mg chl.*a*] $^{-1}$). In order to determine the monosaccharide (MCHO) concentration, the extracted sediment samples were hydrolysed by adding 4 mL of extracted sample with 0.4 mL 1 M HCl to 5 mL glass ampoules. The ampoules were sealed and placed in a heating oven at 100°C for 20 hours. After the hydrolysis process, the content was neutralised with 1M sodium hydroxide (NaOH) and weighed. The sample was then analysed spectrophotometrically as above. The concentration of polysaccharides (PCHO) was calculated by subtracting the MCHO from TCHO.

3.3.8. Statistical analysis

Measured data were compiled in Microsoft Excel and statistical analyses were performed with the computing software, R (R-Core-Team, 2014). The relationship between the measured data and the environmental variables was investigated with Analysis of Covariate (ANCOVA). Additionally, Multivariate Analysis of Variance (MANOVA) was undertaken to study the most significant variation on MPB performance, temporally and spatially. The significance level of $P \leq 0.05$ was considered significant. Non-metric Multidimensional Scaling (MDS) analysis was performed on the data to determine the significance of the different environmental parameters, as well as to investigate the relationships between sites.

3.4. Results

3.4.1. Sampling sites details

Sample collection was carried out during the 2015 monsoon season, which was between May and July. Sampling mainly took place early in the morning, at dawn prior sunrise, at Teluk Bahang, Matang, Gelugor, Jerejak, Gertak Sanggul, and Teluk Air Tawar, and in the evening before sunset at Tanjung Rhu, during the low-tide period (Table 3.4.1).

Sites	Sampling Date	Sampling Time	Weather	Tidal height
Teluk Bahang	7th May 2015	7:30 am	Rained the day before sampling	0.7 m
Teluk Air Tawar	27th May 2015	6:45 am	Rained the day before sampling	1.0 m
Matang	4th June 2015	8:30 am	Sunny with 40% cloud cover	0.7 m
Gelugor	16th June 2015	8:00 am	Raining during sampling period	0.8 m
Jerejak	17th June 2015	7:45 am	Raining during sampling period	0.8 m
Gertak Sanggul	5th July 2015	8:15 am	Raining during sampling period	0.8 m
Tanjung Rhu T1	18th May 2015	5:30 pm	Sunny with 80% cloud cover	0.6 m
Tanjung Rhu T2	18th May 2015	6:30 pm	Sunny with 90% cloud cover	0.5 m
Tanjung Rhu T3	18th May 2015	7:30 pm	Raining during sampling period	0.6 m

Table 3.4.1. The sampling dates and times for each sampling site, as well as the weather condition and tidal heights during the sampling periods.

3.4.2. Environmental variables

As sampling was carried out during the rainy season in Malaysia, the weather was mostly cloudy. Irradiance at these times was relatively low, with the highest irradiance being 200.88 μmol m^{-2} s^{-1} measured at Matang and the lowest being 175.27 μmol m^{-2} s^{-1} at Teluk Bahang (Table 3.2). Sediment surface temperatures varied significantly between sampling sites (p= 3.53 x 10^{-10}, F= 54.83, df= 8), but there were no significant differences between depths within each sampling site (Table 3.4.2). Salinity varied significantly between sampling sites (p=, F=, df= 8), with the highest salinity being 34 ppt (parts per thousand) at Matang, and the lowest, 25 ppt at Jerejak (Table 3.4.2). A significant correlation between salinity and sediment

temperature was observed, where salinity increased with increasing sediment temperature (Fig 3.1).

The porewater nutrient concentrations varied between sampling sites. Nitrate concentrations were about 10 times lower at Teluk Bahang than at other sites (0.05 ± 0.02 mg L^{-1}) (Table 3.4.2). Nitrate concentrations were higher at Tanjung Rhu (0.184 ± 0.050, 0.131 ± 0.049, 0.051 ± 0.020 mg L^{-1} at location 1, 2, and 3, respectively) and lower at Matang (0.010 ± 0.001 mg L^{-1}) (Table 3.4.2). However, Teluk Bahang had significantly higher ammonia concentrations than at other sites (3.049 ± 0.230 mg L^{-1}) (Table 3.4.2). Phosphate concentrations were highest at Gelugor and Jerejak (0.210 ± 0.025 mg/L and 0.212 ± 0.020 mg L^{-1}, respectively) (Table 3.4.2).

3.4.3. Sediment analysis

The sediments at Teluk Bahang, Jerejak, Gelugor and Matang can be categorized as mud with more than half of the total dried sediment consisting of sediments with a grain size smaller than 150 µm (Fig 3.4.1). Tanjung Rhu T1, Tanjung Rhu T3, and Teluk Air Tawar are categorized as sandy mud, as the total dried sediments were dominated by fine sandy clay with some fine sand (Fig 3.1). Tanjung Rhu T2 and Gertak Sanggul were categorized as gravelly, muddy sand, as the sediments were mainly dominated by slightly granular fine sandy mud (Fig. 3.4.1).

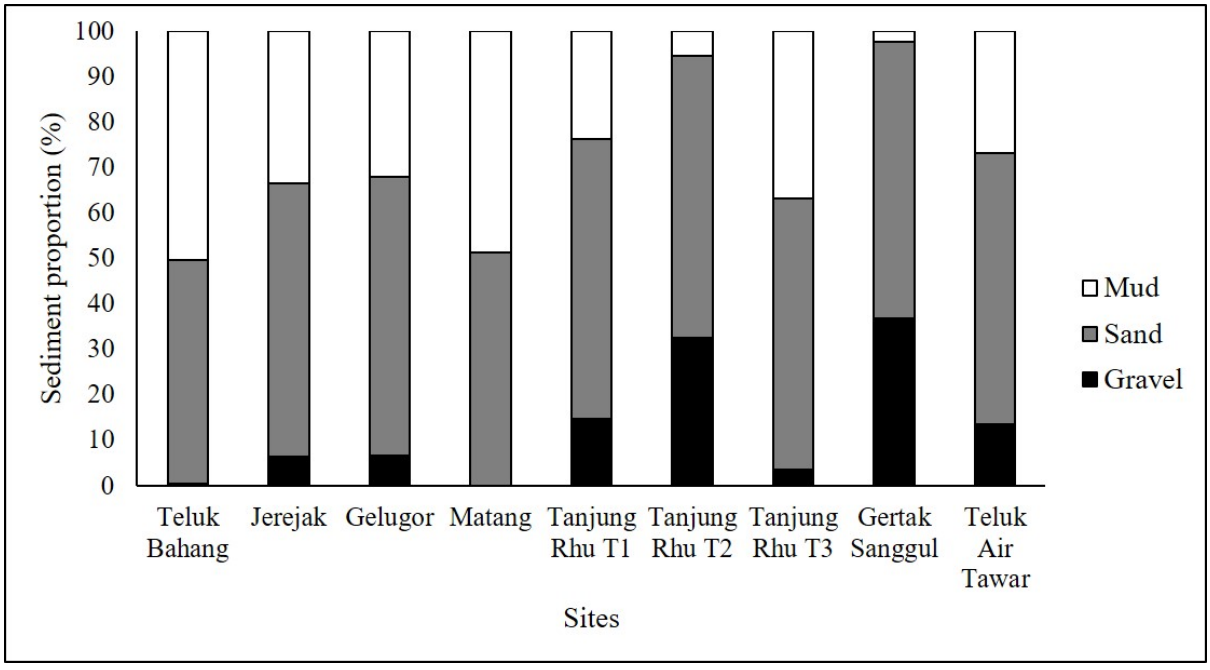

Figure 3.4.1. Sediment composition of each sampling site; white columns represent proportion of sediment with grain size smaller than 90 μm, grey as sediment with grain size between 150 μm and 500 μm, and black as sediment with grain size between 500 μm and 2360 μm.

3.4.4. Species diversity and abundance

Similar dominant species were found among sampling sites, but their relative abundance varied (Fig. 3.4.2). Common species were: *Navicula distans*, *Navicula salinarum*, *Navicula pussila*, *Navicula perminuta*, *Lyrella lyra*, *Cocconeis placentula*, *Navicula algida*, *Fallacia spp.*, *Nitzschia frustulum*, *Achnanthes brevipes*, *Amphora coffeaeformis*, *Petroneis plagiostoma*, *Nitzschia amphibian*, *Diploneis didyma*, *Thalassiothrix spp.*, *Nitzschia panduriformis*, *Pleurosigma salinarum*, *Gyrosigma balticum*, *Surirella fastuasa*, *Trigonium articum*, *Odontella aurita*, *Paralia sulcata*, *Thalassiosira eccentric*, *Coscinodiscus radiates*, and *Skeletonema costatum*. (Fig.3.4.3) (Table 3.4.3). Cells ≤ 10 μm and between 10 and 40 μm were consistently the most common size classes (Fig. 3.4.2).

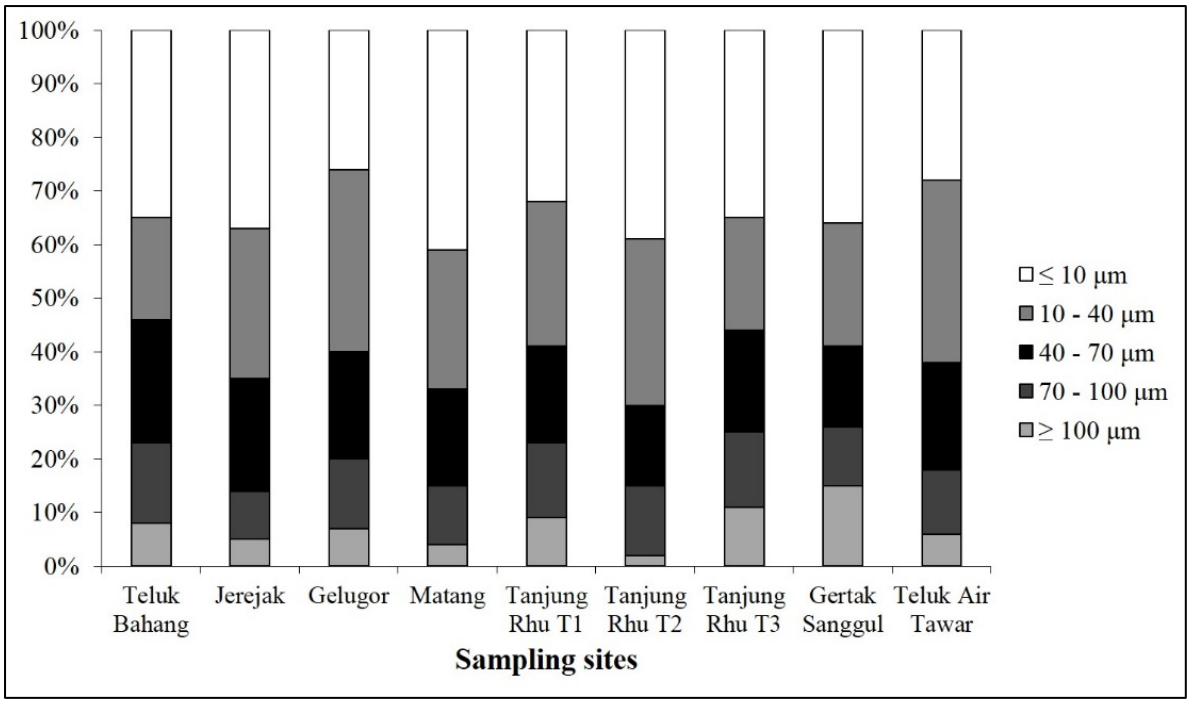

Figure 3.4.2. Relative abundance of MPB diatoms at all sampling sites; categorised by cell size

Table 3.4.2. Irradiances, salinity, sediment temperature (at 3 sampling depths), nutrient concentrations (i.e. nitrate, ammonia, phosphate) at each sampling site.

Sites	Depths (mm)	Parameters					
		Light ($\mu mol\ m^{-2}s^{-1}$)	Salinity (ppt)	Sediment T (°C)	Nitrate (mol/L)	Ammonia (mol/L)	Phosphate (mol/L)
Teluk Bahang	2	175.27 ± 0	33 ± 0	31.0 ± 0	0.031 ± 0.003	0.020 ± 0.002	0.035 ± 0.005
	4			30.8 ± 0			
	6			31.1 ± 2.5			
Tanjung Rhu T1	2	182.41 ± 0	33 ± 0	30.4 ± 2.5	0.184 ± 0.050	0.072 ± 0.024	0.014 ± 0.003
	4			30.2 ± 0			
	6			30.6 ± 2.5			
Tanjung Rhu T2	2	183.59 ± 0	32.3 ± 0.3	30.6 ± 0.2	0.131 ± 0.049	0.025 ± 0.016	0.026 ± 0.004
	4			30.4 ± 0.2			
	6			30.5 ± 0			
Tanjung Rhu T3	2	183.40 ± 0	33.3 ± 0.7	31.4 ± 0.2	0.051 ± 0.020	0.024 ± 0.018	0.018 ± 0.004
	4			31.4 ± 0.2			
	6			31.4 ± 0.2			
Teluk Air Tawar	2	190.45 ± 0	32 ± 0	29.7 ± 0	0.018 ± 0.002	0.004 ± 0.000	0.039 ± 0.002
	4			30.1 ± 2.5			
	6			29.5 ± 0			
Matang	2	200.88± 0	34 ± 0	31.2 ± 0	0.010 ± 0.001	0.065 ± 0.008	0.025 ± 0.003
	4			31.0 ± 2.5			
	6			31.8 ± 0			
Gelugor	2	194.91 ± 2.8	26.8 ± 3.2	29.4 ± 0.2	0.067 ± 0.024	0.054 ± 0.003	0.210 ± 0.025
	4			29.5 ± 0.2			
	6			29.3 ± 0.3			
Jerejak	2	192.02 ± 0.2	25 ± 0	29.2 ± 0.3	0.034 ± 0.013	0.029 ± 0.003	0.212 ± 0.020
	4			29.1 ± 0.2			
	6			29.1 ± 0.2			
Gertak Sanggul	2	182.59 ± 0	27 ± 0	29.8 ± 0.1	0.057 ± 0.003	0.008 ± 0.001	0.092 ± 0.001
	4			29.8 ± 0.2			
	6			29.6 ± 0.2			

1090 Table 3.4.3. Relative MPB species composition (%) at each sampling site.

1091

Species	Teluk Bahang	Tanjung Rhu T1	Tanjung Rhu T2	Tanjung Rhu T3	Gelugor	Jerejak	Matang	Teluk Air Tawar	Gertak Sanggul
Achnanthes brevipes	4.7	6.3	6.7	3.7	2.3	4.3	6.7	4.7	7.0
Achnanthes longipes	2.0	2.7	4.3	2.3	1.0	1.7	3.7	3.3	3.3
Amphora coffeaeformis	3.7	6.7	5.7	6.0	9.0	5.7	0.0	5.0	8.0
Bacillaria spp.	4.0	3.7	0.0	2.7	3.0	4.0	3.0	4.3	2.3
Cocconeis pelta	1.0	0.0	2.7	0.0	1.3	3.3	0.0	0.0	5.3
Coscinodiscus radiatus	4.0	3.0	2.3	3.7	1.7	1.3	2.0	1.7	2.7
Cymbella tumida	0.0	0.0	2.0	0.3	2.0	2.3	1.3	2.7	0.0
Diploneis didyma	0.3	1.3	0.0	1.3	2.3	1.0	0.0	1.7	0.0
Fallacia spp.	2.7	0.0	3.3	0.0	1.7	2.7	0.0	1.3	0.0
Fragilaria schulzii	0.0	0.0	0.0	0.0	5.0	3.7	1.3	3.0	4.3
Gyrosigma balticum	3.7	4.3	1.3	3.0	3.0	2.3	1.7	3.0	3.0
Lyrella lyra	3.0	1.0	2.7	0.7	1.0	2.3	0.0	0.7	0.0
Navicula distans	4.3	2.7	2.3	3.0	2.3	2.3	0.7	2.7	3.7
Navicula menisculus	10.0	8.7	4.7	5.3	11.7	8.7	2.0	6.3	7.3
Navicula perminuta	2.3	2.3	0.3	1.7	3.3	3.7	0.3	1.0	3.0
Navicula pussila	3.0	1.0	0.0	2.0	1.3	0.7	3.0	0.7	2.3
Navicula salinarum	4.3	4.0	3.0	3.7	5.3	6.0	1.3	3.3	5.3

Nitzschia amphibia	4.0	2.3	2.7	4.3	4.3	3.3	4.7	4.7	3.3
Nitzschia panduriformis	2.0	1.7	1.0	2.7	2.3	3.0	2.0	2.0	1.7
Nitzschia transitrans	3.0	2.7	2.0	2.0	2.0	3.7	2.7	2.0	2.7
Odontella aurita	4.0	3.3	5.7	2.7	6.0	4.7	0.0	5.0	0.7
Paralia sulcata	5.7	5.0	0.0	5.7	3.7	3.7	4.0	3.0	7.0
Petroneis plagiostoma	4.3	3.3	3.3	2.3	1.3	2.3	3.7	0.0	1.7
Pleurosigma salinarum	4.7	5.3	2.3	4.7	4.0	3.3	1.7	4.7	4.0
Pseudo-nitzschia spp.	0.7	2.7	0.0	4.0	1.3	0.0	1.3	4.3	4.7
Skeletonema costatum	0.0	15.3	30.3	19.0	1.7	1.0	36.3	14.0	0.0
Surirella fastuasa	2.0	0.0	0.0	0.0	2.7	1.0	0.0	0.0	1.7
Thalassiosira eccentrica	7.3	4.0	3.3	5.7	6.7	8.7	8.3	10.3	9.0
Thalassiosira oestrupii	7.7	5.7	5.3	7.7	5.7	6.0	7.3	3.7	6.0
Thalassiothrix spp.	0.0	0.0	0.0	0.0	1.0	3.0	0.7	0.0	0.0
Trigonium articum	1.7	1.0	2.7	0.0	0.0	0.3	0.3	1.0	0.0

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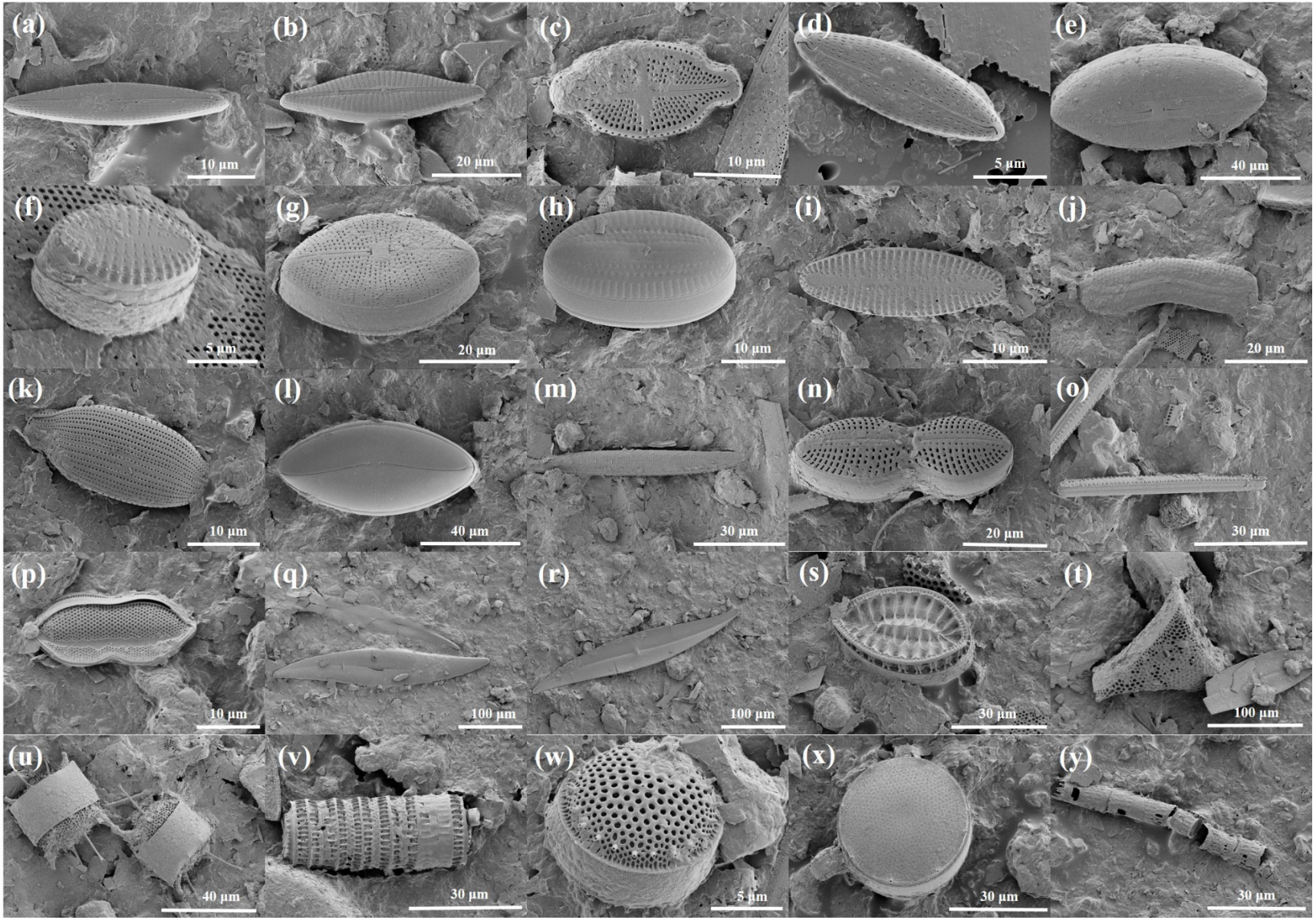
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100 µm
(u)
40 µm
(v)
30 µm
(w)
5 µm
(x)
30 µm
(y)
30 µm

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Figure 3.4.3. SEM photos of the dominant MPB species; *Navicula distans* (a), *Navicula salinarum* (b), *Navicula pussila* (c), *Navicula perminuta* (d), *Lyrella lyra* (e), *Cocconeis placentula* (f), *Navicula algida* (g), *Fallacia spp.* (h), *Nitzschia frustulum* (i), *Achnanthes brevipes* (j), *Amphora coffeaeformis* (k), *Petroneis plagiostoma* (l), *Nitzschia amphibian* (m), *Diploneis didyma* (n), *Thalassiothrix spp.* (o), *Nitzschia panduriformis* (p), *Pleurosigma salinarum* (q), *Gyrosigma balticum* (r), *Surirella fastuasa* (s), *Trigonium articum* (t), *Odontella aurita* (u), *Paralia sulcata* (v), *Thalassiosira eccentric* (w), *Coscinodiscus radiates* (x), *Skeletonema costatum* (y).

3.5.5. MPB chl.a concentrations

MPB chl.*a* varied significantly between sampling sites (Table 3.4). Teluk Air Tawar had the highest chl.*a* concentration, with 37.923 ± 2.333 mg chl. *a* m^{-2}, 14.665 ± 5.667 mg chl.*a* m^{-2}, and 19.452 ± 3.504 mg chl.*a* m^{-2}, at depths of 2 mm, 4 mm, and 6 mm, respectively (Table 3.4.4). Gertak Sanggul had the lowest biomasses with 2.850 ± 0.892 mg chl.*a* m^{-2}, 2.656 ± 0.173 mg chl.*a* m^{-2}, and 1.771 ± 0.181 mg chl.*a* m^{-2}, at depths of 2 mm, 4 mm, and 6 mm, respectively (Table 3.4.4). Additionally, Teluk Bahang and Matang also had relatively higher chl.*a* concentrations (>10 mg chl.*a* m^{-2}), while Tanjung Rhu, Jerejak and Gelugor have relatively lower chl. *a* concentrations (<10 mg chl.*a* m^{-2}) (Table 3.4.5).

ANCOVA results showed that chl.*a* concentration was significantly affected by the sediment temperature, salinity, light intensity and nitrate concentrations (Table 3.4.5). Additionally, the combined effects of sediment temperature, salinity, and irradiance were also significant in affecting the chl.*a* concentrations (Table 3.4.5). Other nutrients such as ammonia, and phosphate did not have significant effects on the chl.*a* concentrations (Table 3.4.5).

Table 3.4.4. ANOVA for chl.*a* and chl.*a*-normalised extracellular carbohydrate of total carbohydrates (TCHO), monosaccharides (MCHO), and polysaccharides (PCHO) between sampling sites and sampling depths, with significant P-values are in **bold**.

Source of variation	df	Sum Sq.	Mean Sq.	F	P-value
Chl.*a*					
Site	8	1552.1	194.01	8.558	**0.000158**
Depth	2	146.1	73.05	3.222	0.0667
MCHO					
Site	8	8.989	1.124	7.478	**0.000349**
Depth	2	0.389	0.195	1.295	0.301
TCHO					
Site	8	9.158	1.145	5.997	**0.00119**
Depth	2	0.345	0.173	0.904	0.425
PCHO					
Site	8	9.845	1.231	5.756	**0.00148**
Depth	2	0.347	0.174	0.812	0.461

Table 3.4.5. The chl.*a* concentrations (mg chl.*a* m^{-2}), phaeophytin concentrations (mg chl.*a* m^{-2}), chl.*a*-normalised TCHO (mg C [mg chl.*a* m^{-2}]$^{-1}$), chl.*a*-normalised MCHO (mg C [mg chl.*a* m^{-2}]$^{-1}$), chl.*a*-normalised PCHO (mg C [mg chl.*a* m^{-2}]$^{-1}$), as well as the percentage of MCHO and PCHO in relation to TCHO.

	Depth	**Chl.*a***	**Phaeophytin**	**TCHO**	**MCHO**		**PCHO**		**MCHO: PCHO**
	(mm)	**Values**	**Values**	**Values**	**Values**	**%**	**Values**	**%**	**Values**
Teluk Bahang	2	21.522 ± 3.850	29.910 ± 8.124	16.310 ± 5.619	4.762 ± 1.293	30.520	11.548 ± 4.334	69.480	0.442
	4	13.752 ± 1.839	23.603 ± 2.222	19.015 ± 3.595	5.850 ± 1.208	30.895	13.165 ± 2.549	69.105	0.450
	6	19.895 ± 4.550	31.681 ± 5.873	15.313 ± 3.279	5.062 ± 0.885	33.833	10.252 ± 2.404	66.167	0.514
Tanjung Rhu T1	2	4.400 ± 0.507	7.479 ± 0.862	18.292 ± 1.040	6.955 ± 0.818	37.901	11.338 ± 0.770	62.099	0.620
	4	3.597 ± 1.226	6.115 ± 2.084	27.167 ± 12.546	9.675 ± 3.512	38.930	17.492 ± 9.045	61.070	0.648
	6	4.2076 ± 1.653	7.150 ± 2.810	12.353 ± 4.093	4.606 ± 1.368	38.725	7.748 ± 2.726	61.275	0.636
Tanjung Rhu T2	2	3.791 ± 1.809	6.444 ± 3.075	39.037 ± 23.975	11.117 ± 6.123	32.618	27.920 ± 17.880	67.382	0.492
	4	6.586 ± 2.255	11.195 ± 3.834	7.754 ± 2.231	2.980 ± 0.898	38.168	4.774 ± 1.334	61.832	0.617
	6	5.838 ± 2.016	9.925 ± 3.427	20.909 ± 12.211	6.550 ± 3.767	31.703	14.359 ± 8.444	68.297	0.464
Tanjung Rhu T3	2	2.546 ± 0.470	4.328 ± 0.800	37.049 ± 7.790	12.302 ± 2.944	32.696	24.747 ± 4.967	67.304	0.488
	4	2.961 ± 0.534	5.033 ± 0.908	49.451 ± 3.631	16.285 ± 1.588	32.854	33.167 ± 2.292	67.146	0.491
	6	1.660 ± 0.166	2.822 ± 0.282	74.216 ± 22.719	24.556 ± 7.531	32.971	49.660 ± 15.198	67.029	0.492
Teluk Air Tawar	2	37.769 ± 2.334	64.208 ± 3.967	2.844 ± 0.447	1.117 ± 0.260	38.364	1.728 ± 0.210	61.636	0.637
	4	14.665 ± 5.667	24.931 ± 9.634	5.896 ± 0.907	2.402 ± 0.607	39.444	3.494 ± 0.300	60.556	0.668
	6	19.452 ± 3.504	33.068 ± 5.957	7.022 ± 1.215	2.617 ± 0.513	36.920	4.405 ± 0.725	63.080	0.588

Matang	2	26.923 ± 4.502	45.769 ± 7.653	13.284 ± 3.148	2.848 ± 0.859	20.516	10.436 ± 2.306	79.484	0.260
	4	10.791 ± 0.848	18.345 ± 1.441	15.645 ± 2.131	3.484 ± 0.674	22.065	12.161 ± 1.549	77.935	0.285
	6	11.704 ± 1.223	19.897 ± 2.079	19.425 ± 4.027	4.582 ± 1.609	21.905	14.843 ± 2.437	78.095	0.288
Gelugor	2	8.716 ± 0.383	14.817 ± 0.652	10.743 ± 1.670	3.113 ± 0.625	28.543	7.629 ± 1.060	71.457	0.401
	4	5.617 ± 0.892	9.459 ± 1.516	16.983 ± 3.804	4.739 ± 1.250	27.532	12.244 ± 2.627	72.468	0.382
	6	5.174 ± 1.397	8.796 ± 2.374	34.369 ± 16.926	9.812 ± 5.293	27.076	24.557 ± 11.633	72.924	0.373
Jerejak	2	4.732 ± 0.706	8.044 ± 1.200	19.856 ± 5.655	6.238 ± 1.973	30.786	13.618 ± 3.682	69.214	0.446
	4	4.317 ± 1.292	7.338 ± 2.197	25.242 ± 11.068	7.657 ± 3.721	29.260	17.585 ± 7.359	70.740	0.415
	6	4.483 ± 0.867	7.620 ± 1.473	29.142 ± 3.053	8.017 ± 0.878	27.515	21.125 ± 2.262	72.485	0.380
Gertak Sanggul	2	2.850 ± 0.892	4.845 ± 1.516	15.633 ± 6.472	3.570 ± 1.351	23.970	12.063 ± 5.223	76.030	0.318
	4	2.656 ± 0.173	4.516 ± 0.294	8.448 ± 2.913	2.402 ± 0.775	28.881	6.046 ± 2.140	71.119	0.407
	6	1.771 ± 0.181	3.010 ± 0.308	12.867 ± 1.716	3.651 ± 0.514	28.574	9.216 ± 1.331	71.426	0.404

1123

Table 3.4.6. ANCOVA results of chl.*a*, chl.*a*- normalised TCHO, chl.*a*- normalised MCHO, chl.*a*- normalised PCHO, F_v/F_m, $rETR_{max}$, E_k, and α in response environmental variables, as well as the combined effects of these variables, where s. temp. is sediment temperature and sal. is salinity. P-values with **bold** represent significant correlations.

Components	Parameters							
	Chl.*a*	**TCHO**	**MCHO**	**PCHO**	**F_v/F_m**	**$rETR_{max}$**	**E_k**	**Alpha**
S. temp.	**0.0322**	**0.0487**	**0.0131**	0.0854	0.180	0.861	0.299	0.0529
Sal.	**0.0395**	**0.0447**	**0.0119**	0.0791	0.144	0.820	0.329	**0.0466**
Light	**0.0320**	**0.0497**	**0.0135**	0.0870	0.181	0.808	0.333	0.0653
S. temp.: sal.	**0.0375**	**0.0447**	**0.0119**	0.0791	0.157	0.791	0.318	0.0509
S. temp: light	**0.0307**	**0.0496**	**0.0134**	0.0869	0.195	0.774	0.324	0.0705
Sal.: light	**0.0379**	**0.0459**	**0.0123**	0.0811	0.156	0.737	0.356	0.0579
S. temp: sal.: light	**0.0360**	**0.0459**	**0.0122**	0.0810	0.170	0.708	0.345	0.0631
Nitrate	**0.0216**	0.628	0.924	0.502	**$1.79x10^{-4}$**	0.686	0.480	**$1.03x10^{-4}$**
Ammonia	0.275	0.313	0.397	0.280	**$8.64x10^{-6}$**	0.962	0.405	**$8.21x10^{-6}$**
Phosphate	0.159	0.396	0.603	0.315	0.0977	0.107	0.647	0.101

3.5.6. MPB extracellular carbohydrate concentrations

As MPB extracellular carbohydrate concentrations are closely related to biomass (Fig. 3.4.4), the extracellular carbohydrate concentrations were normalised to the microphytobenthic chlorophyll concentration and reported as the chl.*a*-normalised carbohydrate (mg C [mg chl.*a* $m^{-2}]^{-1}$). Chl.*a*-normalised TCHO, MCHO, and PCHO values showed significant differences between sampling sites (Table 3.4.4). Tanjung Rhu T3 had highest chl-normalised TCHO, MCHO, and PCHO averaging 53.572 ± 11.38 mg C [mg chl.*a* $m^{-2}]^{-1}$, 17.714 ± 4.021 mg C [mg chl.*a* $m^{-2}]^{-1}$, and 35.858 ± 7.484 mg C [mg chl.*a* $m^{-2}]^{-1}$, respectively. Teluk Air Tawar had the lowest chl-normalised TCHO, MCHO, and PCHO, averaging 5.254 ± 0.856 mg C [mg chl.*a* $m^{-2}]^{-1}$, 2.045 ± 0.460 g glucose [mg chl.*a* $m^{-2}]^{-1}$, and 3.209 ± 0.412 mg C [mg chl.*a* $m^{-2}]^{-1}$, respectively (Table 3.4.5). PCHO concentrations were higher than MCHO and contributed more than half of TCHO in all sampling sites (Table 3.4.5). The ratio of MCHO: PCHO further showed that PCHO concentrations almost tripled MCHO concentrations at each depth at all sampling sites (Table 3.4.5).

ANCOVA results showed that chl.*a*-normalised TCHO and MCHO were significantly affected by sediment temperature, salinity and irradiance. Furthermore, the combined effects of these environmental variables were also significant in affecting the TCHO and MCHO concentrations (Table 3.4.6). However, neither individual factors, nor the combined factors contributed a significant effect on PCHO (Table 3.4.6). Nitrate, ammonia, and phosphate concentrations also did not have significant effects on the chl-normalised extracellular carbohydrate components (Table 3.4.6). Multivariate analysis further showed that the variations in extracellular carbohydrate concentrations were mainly site-specific and that sediment temperature, salinity, and irradiance had significant influences on extracellular carbohydrate production (Fig. 3.4.5). Sediment temperature was the factor most likely to cause the variation (Fig. 3.4.5).

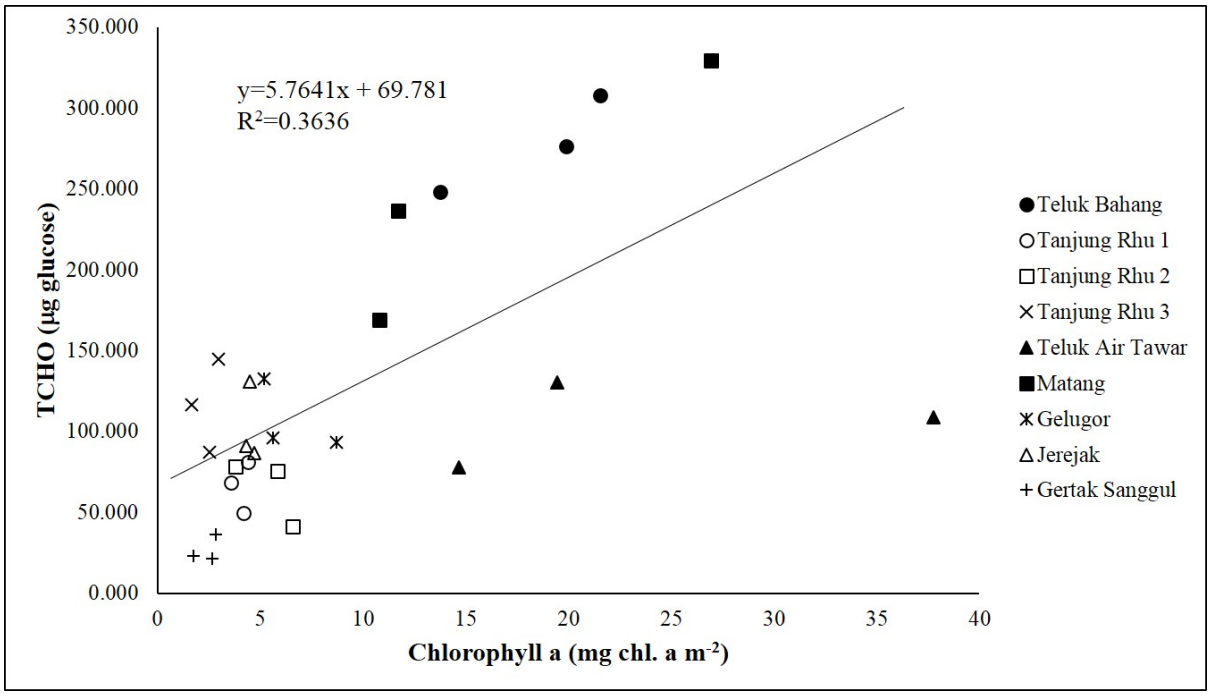
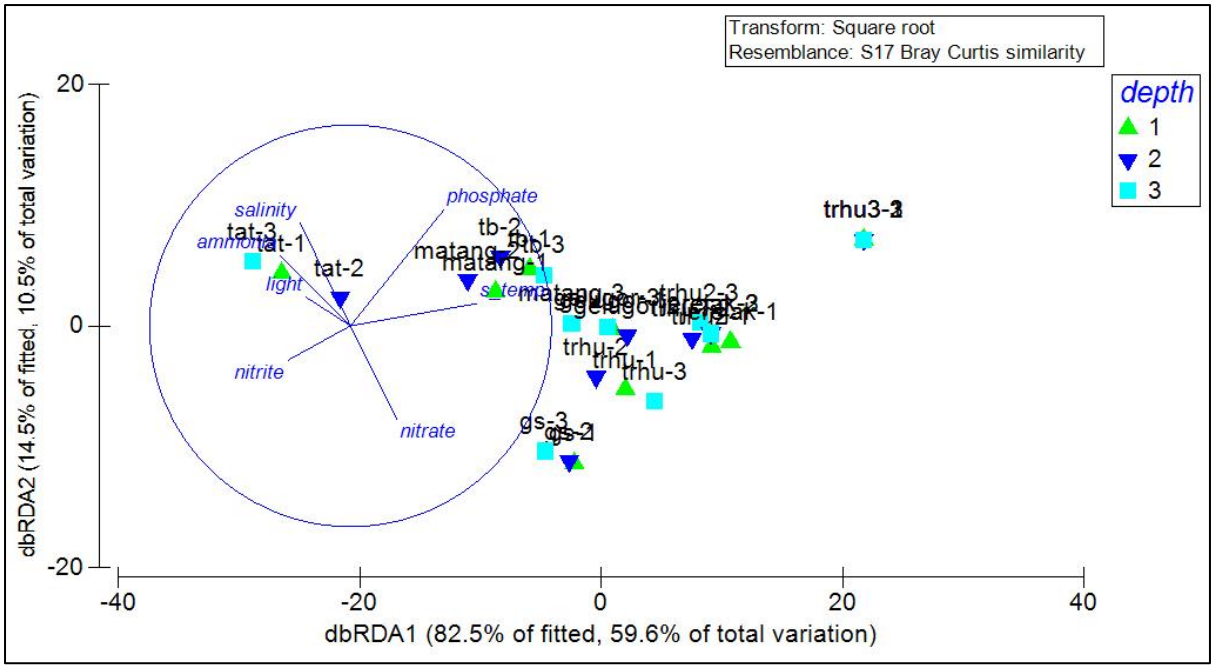


Figure 3.4.4. Plot of TCHO values against chl.*a* concentration at all sampling sites.



1156

1157 Figure 3.4.5. Multivariate analysis (Bray Curtis Similarity) of TCHO (-1), MCHO (-2), and
1158 PCHO (-3) for all depths (1= surface, 2 = 3 cm, 3 = 5cm) at all sampling sites (Tanjung Rhu =
1159 trhu, Teluk Air Tawar = tat, Gertak Sanggul = gs, Teluk Bahang = tb).

1160 *3.5.7. Photosynthetic performance*

1161 There were no significant differences in F_v/F_m, $rETR_{max}$, and E_k between sampling depths,
1162 except α (p= 0.0394, F= 3.891, df= 2) (Table 3.4.7). Highest F_v/F_m values were at Teluk Bahang,
1163 while lowest values were at Teluk Air Tawar (Table 3.4.7). Highest $rETR_{max}$ values were at
1164 Teluk Bahang, while lowest values were at Gertak Sanggul (Table 3.4.7). Highest E_k values
1165 were at Tanjung Rhu T3, while lowest values were at Gertak Sanggul (Table 3.4.7). Highest α
1166 values were at Teluk Bahang, while lowest values were at Jerejak (Table 3.4.7). In general, the
1167 photosynthetic performance of MPB from all sampling sites was below an average healthy
1168 photosynthetic performance, as their photosynthetic efficiency (F_v/F_m) values from all sites
1169 were lower than 0.4 (Table 3.4.7).

1170 ANCOVA results showed that only α was affected by salinity (Table 3.4.6). Furthermore, the
1171 combined effects of these environmental variables did not impact the photosynthetic
1172 performance significantly (Table 3.4.6). Nutrient concentrations had more effect on the MPB
1173 photosynthetic performance than chl.*a*, TCHO, MCHO, and PCHO. Only F_v/F_m and α
1174 significantly affected by nitrate and ammonia (Table 3.4.6). Additionally, nutrient
1175 concentrations did not have a significant impact on the $rETR_{max}$.

Table 3.4.7. Chlorophyll and chlorophyll-normalised extracellular carbohydrate concentrations, as well as photosynthetic parameters such as F_v/F_m, $rETR_{max}$, E_k (μmol photon $m^{-2}s^{-1}$), and α values measured at each sampling at 3 different depths.

Sites	Depths (mm)	F_v/F_m	$rETR_{max}$	E_k	α
Teluk Bahang	2	0.344 ± 0.046	81.149 ± 3.159	275.861 ± 42.260	0.320 ± 0.059
	4	0.389 ± 0.072	105.719 ± 7.653	369.297 ± 108.368	0.360 ± 0.096
	6	0.347 ± 0.101	87.149 ± 3.457	427.612 ± 195.739	0.353 ± 0.112
Tanjung Rhu 1	2	0.255 ± 0.081	70.440 ± 9.850	309.044 ± 77.657	0.268 ± 0.062
	4	0.359 ± 0.089	52.846 ± 5.314	196.682 ± 38.640	0.312 ± 0.081
	6	0.270 ± 0.081	60.411 ± 9.029	234.687 ± 34.222	0.276 ± 0.061
Tanjung Rhu 2	2	0.256 ± 0.008	49.030 ± 2.189	163.364 ± 5.205	0.302 ± 0.023
	4	0.319 ± 0.011	37.519 ± 5.676	138.382 ± 30.655	0.282 ± 0.019
	6	0.307 ± 0.035	41.414 ± 10.512	142.979 ± 23.702	0.275 ± 0.023
Tanjung Rhu 3	2	0.231 ± 0.034	81.407 ± 19.343	400.866 ± 72.644	0.210 ± 0.012
	4	0.227 ± 0.019	74.718 ± 9.169	382.747 ± 28.253	0.193 ± 0.010
	6	0.170 ± .032	84.917 ± 3.055	416.128 ± 49.666	0.213 ± 0.026
Teluk Air Tawar	2	0.179 ± 0.028	64.912 ± 6.118	335.002 ± 25.980	0.198 ± 0.023
	4	0.199 ± 0.007	70.817 ± 5.331	325.474 ± 23.859	0.217 ± 0.003
	6	0.161 ± 0.022	75.117 ± 10.088	346.892 ± 46.457	0.217 ± 0.015
Matang	2	0.234 ± 0.032	59.788 ± 7.034	254.275 ± 16.095	0.233 ± 0.014
	4	0.162 ± 0.027	54.027 ± 12.854	332.119 ± 96.608	0.170 ± 0.009
	6	0.158 ± 0.032	56.597 ± 4.951	359.676 ± 50.744	0.162 ± 0.011
Gelugor	2	0.365 ± 0.023	35.339 ± 1.678	110.652 ± 2.980	0.337 ± 0.023
	4	0.244 ± 0.057	30.742 ± 3.367	189.850 ± 27.426	0.215 ± 0.042
	6	0.216 ± 0.030	32.454 ± 1.761	174.148 ± 18.318	0.218 ± 0.031
Jerajak	2	0.217 ± 0.031	45.531 ± 9.559	289.830 ± 34.476	0.153 ± 0.018
	4	0.226 ± 0.024	40.926 ± 2.714	280.419 ± 15.410	0.146 ± 0.005
	6	0.283 ± 0.015	43.172 ± 8.478	260.119 ± 30.402	0.163 ±0.017
Gertak Sanggul	2	0.244 ± 0.021	28.045 ± 1.520	103.529 ± 2.509	0.271 ± 0.015
	4	0.264 ± 0.020	39.109 ± 7.727	137.390 ± 35.950	0.298 ± 0.027
	6	0.229 ± 0.018	20.947 ± 3.557	89.045 ± 17.381	0.238 ± 0.009

3.6. Discussion

Sampling for this study occurred during the Malaysian dry season. However, heavy cloud cover in this period resulted in a reduction in light intensities, usually to less than 250 μmol m^{-2} s^{-1}. Furthermore, there was a short raining period during this dry season, resulting in the freshwater input from the rain, which impacted average porewater salinity. The relatively low sediment temperatures that were observed for a tropical country were due to the combined effects of lower temperature rainwater and thick attenuating cloud cover

Phosphate concentrations increased with increasing salinity, implying that seawater was the primary source of phosphate in the estuaries of the western Peninsular Malaysia. There were no significant variations in MPB species composition between sediment depths, probably due to sediment resuspension by the high input of freshwater as well as wave action. Additionally, reduced irradiance likely prevented the MPB from migrating into deeper sediment, resulting in a more homogeneous surface community.

Chl.*a* and extracellular carbohydrate concentrations are potential indicators of MPB biomass (Thornton et al., 2002). Variations in chl.*a* concentrations between sites were mainly due to:

1) differences in the sediment grain size, and

2) differences between the environmental variables such as sediment temperature, irradiance and salinity.

Lower chl.*a* concentrations were found at the sandier sites (Tanjung Rhu 1, Tanjung Rhu 2, Tanjung Rhu 3, and Gertak Sanggul), while higher chl *a* concentrations were found at muddier sites (Teluk Bahang, Teluk Air Tawar, and Matang). Non-cohesive sediments (i.e., sand) usually reportedly contains lower MPB biomass than silts or muds, as these sediments usually have less species composition (Underwood and Kromkamp, 1999; Underwood and Smith, 1998; Daniel et al. 2002). The ANCOVA results further showed that chl *a* concentration was not only affected by differences in grain size but also by sediment temperature, salinity and irradiance (Table 3.5). Salinity has been recognised as a major factor effecting the distribution of MPB, where different species of benthic diatoms have different favourable salinity range for themselves (Cox, 1995; Underwood and Provot, 2000). Other important factors include temperature and dissolved inorganic nitrogen (DIN) (Thornton et al. 2002). The combined effects of these factors also effected the chl.*a* concentrations in the present study (Table 3.5).

Many of the important environmental variables affecting MPB are usually interrelated and co-vary and this often makes it difficult to isolate individual effects (Brotas et al., 1995). Irradiance, sediment temperature and salinity are usually closely related; high irradiances are associated with cloud free conditions and greater temperatures leading to increases in sediment temperature, which increases the rate of evaporation and desiccation in the sediment. This causes an increase in sediment pore water salinity. However, this study was undertaken during the rainy period of dry season, when maximum rainfall occurs. This, lower salinity, resulting from high rainfall, was responsible for lower chl.*a* concentrations at Gelugor, Jerejak, and Gertak Sanggul (Table 3.4). Sediment grain size also serves as a reliable predictors of MPB species composition and abundance (Mitbavkar and Anil, 2006). Here, a higher abundance of motile diatoms such as *Navicula spp.*, *Pleurosigma spp.*, *Gyrosigma spp.*, *Cocconeis spp.*, *Amphora spp.* and *Nitzschia spp.* were found at the sandier sites, such as Teluk Air Tawar, Tanjung Rhu, Jerejak and Gelugor, while diatoms such as *Thalassiosira spp.*, *Skeletonema spp.* and *Odontella spp.* were more abundant in the muddier sites such as Teluk Bahang and Matang due to resuspension (Table 3.3). The PCO analysis showed that the spatial variation in species composition between sampling sites was 39.3% but up to 26.8% vertically (i.e., within the sediment). So, although chl.*a* concentrations varied significantly between sampling sites, there were no significant differences between depths within each sampling site (Table 3.3). This vertical uniformity may have been caused by additional physical mixing during the wet season (Thornton et al., 2002). Furthermore, benthic chl.*a* concentrations were found to be distributed into the deeper depths of the sediment during monsoon season, while concentrated in the surface sediment during the pre- and post-monsoon seasons (Mitbabavkar and Anil 2006, 2008).

There is usually a close relationship between MPB biomass and extracellular carbohydrate at the sediment surface of temperate estuaries (Underwood and Smith, 1998; Blanchard et al., 2000; de Winder et al., 1999; van Duyl et al., 1999; Yallop et al., 2000).) (Fig 3.3). Here, as with chl.*a* concentration, significant differences were found between sites for chl.*a*- normalised extracellular carbohydrate concentrations (Table 3.4). Lower chl *a*-normalised TCHO were found at the sandier sites, presumably because it is more easily washed away by currents and tidal immersion. As this study was performed during the rainy period of wet season, excessive rainfall could have been responsible for the rapid removal of the extracellular carbohydrates. Another possibility of lower TCHO at the sandier sites may be due to lower production of TCHO for migration purposes.

The measured chl *a*- normalised TCHO and MCHO concentrations were significantly affected by salinity, sediment temperature and irradiance, but not nutrient concentrations. Elevated nutrient concentrations, associated with low salinity, low phytoplankton biomass, low bacterial cell numbers occurring during monsoons season has been observed previously in the tropical Mandovi Estuary (Khodse et al., 2010). However, in this system, it was associated with higher concentration of TCHO (Pratihary et al., 2009). Meon and Kirchman (2001) showed that distribution of TCHO is affected by several factors including salinity, nutrient, MPB biomass and composition, and bacterial abundance. Additionally, nutrient limitation was found to increase the amount of colloidal carbohydrate and EPS produced by diatoms (Staats et al., 2000a, Smith and Underwood, 2000). In contrast, nutrient concentrations in this study did not have a significant impact on the TCHO, MCHO, and PCHO.

In the present study, irradiance was the environmental variable most strongly affecting MPB carbohydrate standing stocks. Higher chl.*a*-normalised extracellular carbohydrate was measured at Tanjung Rhu, as sampling and measurements were made at dusk. Here the MPB had already been exposed to higher irradiance during low-tide and the extracellular carbohydrates were likely to have been produced as photosynthetic overflow, or for vertical migration. Chl.*a* concentrations, however, were not high, and they were homogeneous throughout the sampling depths, hence vertical migration was unlikely to be undertaken by the MPB (Table 3.4.5). Tropical MPB biofilms often have higher ratios of colloidal carbohydrate to chl*a* than temperate estuarine biofilms. This is probably due to a combination of high irradiance and low nutrient availability that leads to photosynthetic overflow (Underwood, 2002).

In the present study, there were significantly higher concentrations of PCHO than MCHO and PCHO accounted for more than 60% of TCHO at all sites. PCHO has also been found to be the most abundant constituent of TCHO in other tropical marine studies (Hung et al., 2003, Wang et al., 2006). Khodse et al. (2010) found that the ratio of PCHO to TCHO was higher during the monsoon season in Mandovi estuary, which they suggested may have been caused by elevated extracellular carbohydrate production by MPB and/or additional organic matter from the river run-off. TCHO may serve as a source of carbon and energy for the heterotrophic activity during the monsoon season. Water temperature effects both bacterial abundance and microbial activity and at elevated temperatures they preferentially utilise specific and more labile DOC compounds (Apple et al., 2006; Hung et al., 2003).

In the present study, MCHO accounted for a relatively low proportion of TCHO (20-40%). Similar results were reported from coastal and oceanic waters of Mexico (Hung et al., 2003). The MCHO:PCHO ratio varied between sampling sites; reflecting the impact of the environment as optimum environmental conditions produce a higher MCHO:PCHO ratio (Smith et al., 1992; Meon and Kirchman, 2001). High MCHO: PCHO ratios typically indicate less variability and greater biomass during the pre-monsoon season. Lower concentrations of MCHO were probably due to low rates of heterotrophic decomposition (Agogué et al., 2014; Miyatake et al., 2014).

There were no significant relationships between MPB photosynthetic performance and sediment temperature, irradiance, or salinity. This is most likely due to the acclimation of the MPB communities in response to the low light monsoon season. MPB photosynthetic performance is usually correlated with dissolved inorganic nutrient concentrations (Underwood, 2002; Geider et al., 1997; Kromkamp et al., 1998)). In this study, nutrient levels seemed to have had a greater impact on MPB photosynthetic performance than other major environmental variables (i.e., sediment temperature, light and salinity). Nitrate and ammonia had significant effects on F_v/F_m and α, while nitrate had a significant impact on E_k, (Table 3.5). Effects were mostly site-specific; Teluk Bahang and Tanjung Rhu had much higher concentrations of ammonia and nitrate, respectively, compared to other sites (Table 3.2).

There is usually a close relationship between MPB acclimation and nutrient status (Underwood, 2002, Underwood et al., 1995). Cook et al. (2007) further found that MPB had better photosynthetic performance in habitats with high nutrient concentrations, even though they had to withstand greater extremes of sediment temperature, irradiance and salinity. Under these conditions, larger, motile diatoms are able to avoid photo damage by vertical migration (Underwood, 2002). However, the MPB communities from the current study were more homogenised due to their constant resuspension by wave action and rainwater input (initial microscopic observation at different depths). In this study, both E_k and $rETR_{max}$ values did not show a significant response to changes in nutrient levels at any site, although significant effects of nitrate and phosphate were observed on F_v/F_m and α. E_k values at the sandier sites may be overestimates, because backscatter by the coarse sands can increase the *in situ* irradiance received by the cells (Kuhl and Jorgensen, 1994). Conversely, at muddier sites, more irradiance would can be absorbed by dissolved substances before impacting on MPB, which would result in an underestimation of E_k values (Underwood, 2002). As a result, MPB during this sampling

period is potentially more likely to experience photoinhibition, as they are adapted to seasonal lower irradiances but will still experience sporadic higher irradiances. Although motile diatoms can migrate vertically throughout the sediment to 'behaviourally' photoacclimate (Kromkamp et al., 1998; Paterson et al., 1998; Perkins et al., 2002; Perkins et al., 2001; Underwood and Kromkamp, 1999), resuspension of sediments and cells results in a vertically homogenised MPB photosynthetic performance.

3.7. Conclusion

In conclusion, MPB properties including chl.*a* concentrations, extracellular carbohydrate concentrations, as well as photosynthetic performance varied significantly between sampling sites, but differences between depths were not significant. This may be due to the higher hydrodynamic forcing at these sites during monsoon season increasing the rate of resuspension and resulting in a more homogenised composition throughout sampling depths. MPB showed relatively low chl.*a* concentrations during the monsoon season. The sediment temperatures, irradiances and salinities all played crucial roles in affecting the chl *a* and extracellular carbohydrate concentrations at each site. Furthermore, the combined effects of these factors were significant. However, nutrient concentrations did not have a significant impact on either chl.*a* concentrations nor extracellular carbohydrate concentrations. The measured photosynthetic performance showed that the MPB were experiencing some level of stress. The photosynthetic performance was not affected by the sediment temperature, salinity, and irradiance, but F_v/F_m and α were affected by nitrate and ammonia concentrations. This study was carried out during the rainy period of dry season and the key environmental variables, such as salinity, sediment temperature, and irradiances, can be expected to be lower compared to those during other periods of the season. This changed in the environmental variables may have driven the MPB into a "stressed" condition. Furthermore, sudden changes in nutrient levels caused by rainfalls prior to samplings also may have had a greater impact on the MPB photosynthetic performance, which compounded the identification of the effects of other environmental variables.

Chapter 4: Seasonal dynamics of extracellular carbohydrate composition in a southern temperate estuary

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4.1. Abstract

Microphytobenthos (MPB) are unicellular microorganisms that inhabit the photic zone of tidal flats and play a significant role in coastal primary production. MPB communities are mainly comprised of diatoms, with small proportions of dinoflagellates and euglenoids. These microorganisms are highly sensitive to the surrounding environments and use different mechanisms in response to rapid change. Production of extracellular carbohydrate, which contains a high concentration of glucose, is one of the mechanisms they employ for survival.

Neither the chl *a*-normalised glucose concentration nor the relative proportions of the different monosaccharides, varied between sampling depths at both Penna Beach and Kings Beach. Glucose concentration varied significantly between sampling sites, with glucose concentrations at Penna Beach much higher (>0.1 mg/mL) than those of Kings Beach (<0.05 mg/mL). Glucose concentrations also varied significantly between seasons, being highest during spring and lowest during winter at Penna Beach, but this was not observed at Kings Beach. Concentration of other monosaccharides remained uniformly low.

That there were no significant differences in colloidal carbohydrate composition with depth suggests that either the MPB are actively migrating through the top 5 mm or that there is significant bioturbation. Although glucose concentrations varied significantly between seasons at Penna Beach, the changes were not correlated with any measured environmental factors. Therefore, the elevated glucose concentration may due to other reasons such as hydrolysis by heterotrophic bacteria or higher production of glucan as a photosynthetic storage product for dark survival and salinity during spring.

4.2. Introduction

Tidal flats make a vital trophic contribution in the productivity of many coastal ecosystems. These systems are highly productive, largely due to the microorganisms that inhabit the photic zones of the sediment. These microorganisms are known as microphytobenthos (MPB) and are mainly comprised of pennate diatoms (*Bacillariophyceae*) (Round et al., 1990, Naoshige et al., 1999, Underwood and Kromkamp, 1999), with small proportions of dinoflagellates and euglenoids. MPB occurs in most aquatic environments and is often the main primary producer on intertidal mudflats, although often showing considerable patchiness in biomass distribution (Taylor et al., 1999, Smith and Underwood, 1998).

Although intertidal zones are highly dynamic, due mainly to rapid physical and chemical changes, their productivity is high. MPB have evolved a range of mechanisms to respond to these changes, both behaviourally (Serôdio et al., 1997) and physiologically (Blanchard et al., 2004, Vieira et al., 2013, van Leeuwe et al., 2008). Physiological responses include photoacclimation, i.e. changing their photosynthetic performance in response to changes in light (Blanchard et al., 2004; Dodds et al., 1999; Guarini et al., 1997; Kromkamp et al., 1998; Yun et al., 2010; Lee and McMinn, 2013). Behavioural responses include vertical migration, whereby an optimum depth in the sediment is selected to prevent photodamage (de Brouwer and Stal, 2001; 2002; Goto et al., 2001; Perkins et al., 2001; Smith and Underwood, 1998; Stal, 2003; Stal and Défarge, 2005; Underwood and Paterson, 2003; Underwood and Smith, 1998). This motility is essential to enable diatoms to migrate into the photic zone of the sediment during times of emersion (Cohn and Disparti, 1994). MPB show rhythmic migrations in response to both diel light and tidal cycles (Happey-Wood and Jones, 1988; Serôdio et al., 1997), the amplitude of which has been reported to vary from 1.6 mm (Paterson, 1986) to 12 cm (Kingston, 1999). MPB migrate deeper into the sediment during the high-tide period to prevent disturbance by wave action. Although migration occurs mostly in the only the top few millimeters, light penetration is often less than 0.5 mm and therefore, even during the day (Underwood and Kromkamp, 1999), photosynthesis is not possible at greater depths (Garcia-Pichel and Bebout, 1996). The vertical migration of diatoms is possible through the exudation of copious amounts of polysaccharides, called extracellular polymeric substances (EPS), through the ‘raphe’, This EPS, which is composed mainly of carbohydrates (80%, Myklestad 1974) (Hoagland et al., 1993), including polysaccharides, is important for bacterial metabolism (Decho and Lopez, 1993), sediment stabilisation (de Brouwer et al., 2000; de Brouwer and Stal,

2001), facilitating sessile adhesion of the cell to a substratum (Lind et al., 1997; Wetherbee et al., 1998), resistance to metal toxicity, and desiccation resistance (Decho and Lopez, 1993, Hoagland et al., 1993, Paterson, 1986, Paterson et al., 2000, Sutherland, 2001). In addition to carbohydrates, the EPS matrix also contains a wide variety of proteins, lipids, and other more surprising compounds such as nucleic acids. This very complex composition makes biochemical characterisation particularly difficult due to the diversity in sugar monomers, linkages, low concentrations, and the interactions of compounds during assays.

Carbohydrates that are easily extracted from sediments with water have been termed "colloidal carbohydrates" (Underwood et al., 1995) and include polymeric forms (EPS) and simple sugars. Colloidal carbohydrate is commonly used in the quantification of extracellular carbohydrates and usually shows a significant correlation to chlorophyll a (chl *a*) in intertidal mudflats that are dominated by diatom assemblages (Underwood and Smith, 1998; Blanchard et al., 2000). Further hydrolysis procedures of sediment samples allow measurements of total carbohydrate content (Taylor and Paterson, 1998; Taylor et al., 1999).

Production of extracellular carbohydrates has been observed to occur when the sediment is exposed to the light (de Winder et al., 1999; Staats et al. 2000b; Taylor et al., 1999) and is dependent on a number of factors including time of exposure (Underwood and Paterson, 1993), growth stage (Smith and Underwood, 1998; Staats et al., 1999) and morphology of the mudflat (Blanchard et al., 2000; Taylor and Paterson, 1998). Furthermore, increased production of extracellular carbohydrates has been observed concurrent with changes in diatom numbers at the mudflat surface, typically at the start and end of the emersion period (Smith and Underwood, 1998; Underwood and Smith, 1998).

There are several factors that may control exopolysaccharide production. For instance, a previous investigation by Staats et al., (1999) demonstrated that oxygenic photosynthesis was required for polysaccharide secretion in the diatom *Cylindrotheca closterium* (Ehrenb.) Reimann and Lewin. It has also been shown that EPS can be produced as a result of unbalanced growth, arising from factors such as at high irradiances and/or low nutrient availability (de Brouwer and Stal, 2001; Waite et al., 1995; Staats et al., 2000a). However, little is still known about the effects of nutrient availability on polysaccharide secretion by epipelic diatoms. It has been generally assumed that growth in sediments is not limited by nutrients, because nutrient concentrations in pore waters are generally high (Cadée and Hegeman, 1974; Admiraal et al., 1982; Sundby et al., 1992). However, in the thin layer of cells at the sediment surface, biomass

may be highly concentrated, and therefore nutrients may temporarily become depleted (Admiraal et al., 1982). Indeed, evidence is accumulating that at high population densities, growth of benthic diatoms may become nutrient limited, especially by nitrogen (Hillebrand and Sommer, 1997; Cook et al. 2004).

Previous studies have shown little correlation between bacterial densities and algal biomass (Cole et al., 1988, Underwood and Paterson, 1993, Underwood and Paterson, 2009). Although some studies have found that the abundance of bacteria in sediment is correlated with primary production (Cammen and Walker, 1986), others have found that particle size, organic content, depth of the redox-discontinuity layer, and depth in the sediment are more important factors determining bacterial distribution (Dale, 1974; DeFlaun and Mayer, 1983; Novitsky, 1987). A proportion of the bacteria in the sediment may be dormant and high bacterial densities do not necessarily indicate high bacterial activity (Carman, 1990; Parkes and Taylor, 1985). The role of bacteria in producing and utilizing exopolymers in sediments and the effect this may have on stability and productivity is still largely unknown (Yallop et al., 2000). Through the excretion of EPS, diatoms are responsible for a considerable input of 'high-quality' organic carbon into the sediment that may subsequently be utilized as a food source for heterotrophic consumers (van Duyl et al., 1999).

In situ studies have so far only reported on daytime emersion periods (Taylor et al., 1999) or have lacked sufficient vertical resolution to be able to compare day and night-time emersion periods (Staats et al., 2000b; van Duyl et al., 1999). There is also very little information on the biochemical properties of extracellular carbohydrates in mudflat sediments. Information about the monosaccharide composition of carbohydrates has mainly come from work on cultures (de Winder et al., 1999; Staats et al., 1999), although Taylor and Paterson (1998) analyzed the monosaccharide composition of colloidal and bulk carbohydrates from the Eden estuary, Scotland. Several biochemical studies have assessed the carbohydrate composition of planktonic samples and diatom mono-cultures. In these studies, carbohydrates were isolated from the culture medium by lyophilisation and were generally found to contain galactose, fucose and rhamnose as major components, while mannose, glucose, xylose and arabinose were present to a lesser degree (Myklestad and Haug 1972; Smestad et al., 1974). Glucose in particular, was found to be one of the most abundant sugars in extracted MPB extracellular carbohydrate. In previous studies, glucose was found to be excreted by MPB as 'photosynthetic overflow' in response to unfavourable conditions, such as nutrient limitation and/or excessive

irradiances (Staats et al. 1999; Underwood and Paterson, 2003; Cook et al., 2004; 2007). Additionally, in some MPB species, glucose is stored as glucan to provide energy for the cells during night time or under dark conditions. Glucose is also the preferred sugar to be consumed by bacteria, as other extracellular carbohydrates have a higher complexity (Goto et al. 2001) and so are more difficult to break down. Bacteria take up these more complex carbohydrates by first hydrolysing them into simpler sugars, particularly glucose (Agogué et al., 2014).

A large number of studies have examined the fine structure, chemical components and functions of EPS (Hoagland et al., 1993). The dynamics of extracellular carbohydrates in intertidal mudflats has also been studied at different temporal and spatial scales (Underwood and Paterson, 1993; Taylor and Paterson, 1998; Blanchard et al., 2000; Staats et al., 2000b; de Brouwer et al., 2000). In addition to species compositional data, sediment size-distribution analyses can provide information on the properties of extracellular carbohydrates and may therefore be useful in understanding the dynamics of these sugars in intertidal mudflats.

The seasonal MPB production of total carbohydrates (TCHO), bound monosaccharides (MCHO and polysaccharides (PCHO) was discussed in Chapter 2 (herein). In that study the concentration of colloidal monosaccharides was too low to be measured by the spectrophotometric method of Myklestad et al. (1997). Here, more sensitive HPLC and LP-MS methods are used to specifically quantify colloidal monosaccharide concentration. The primary aim was to follow the fate of extracellular monosaccharides by examining changes in its composition with depth over time. It was also hypothesised that changes in glucose production, as photosynthetic ‘overflow’, would occur in response to unfavourable environmental conditions such as higher irradiance, sediment temperature, or salinity.

4.3. Materials and Methods

4.3.1. Sample collection

The sediment cores were collected from Penna Beach (42° 47’ 06"S, 147° 31’ 13"E) in Pittwater Reserve and Kings Beach (42° 53’ 44"S, 147° 20’ 01"E) in Errol Flynn Reserve, Sandy Bay, during each season between April 2014 and January 2015 (Fig. 4.3.1). The cores were taken during the end of the low-tide period. Sediment cores were collected by inserting a 45 mm perspex core into the sediment. The cores were sectioned into 1 mm intervals and the samples from 1 mm, 3 mm, and 5 mm were selected for further analysis.

Figure 4.3.1. Sampling sites that were chosen in the greater Hobart Area, Tasmania for the current study.

4.3.2. Environmental variables

The irradiance and salinity at each sampling site were measured using a Biospherical QP radiometer with 2π sensor, and refractometer (Hanna Instruments, H96822, Woonsocket,

Rhode Island, USA), respectively, during each sampling. Vertical temperature profile within the sediment was measured by inserting a temperature probe (HI 766C, K-type thermocouple thermometer, HI935005, Hanna Instruments, Woonsocket, Rhode Island, USA) vertically into the sediment. Measurements were taken at 1 mm interval, down to 5 mm. Other environmental parameters such as rainfalls, wind speed and direction, and humidity were obtained from the Australian Bureau of Meteorology. Sediment grain size was analysed at both sampling sites using standard sieving methods.

Relative species abundance was determined for both sites, following the approach introduced in Chapter 2 (herein). Samples were preserved in Lugol's iodine solution prior to preparation for Scanning Electron Microscopy (SEM) analysis. Hydrogen peroxide (H_2O_2) was used to clean the preserved samples of organic material. The samples were then filtered on a 2 µm membrane filter and mounted on SEM stubs with double adhesive tape. The stubs were examined using Hitachi SU-70 Analytical Field Emission Scanning Electron Microscope (FE-SEM) (Hitachi, Naka Division, Ibaraki Prefecture) and species identification was performed following Saunders et al. (2010).

4.3.3. Sample preparation for chromatography analyses

Triplicate sediment samples were collected and kept in ice box filled with dried ice and then were brought back to laboratory immediately. Upon returning to the laboratory, the colloidal carbohydrates were extracted from the sediment samples with 10 mL of milli Q (MQ) water at 30 ˚C for an hour. The dissolved colloidal carbohydrates were filtered through 0.25 µm filters into 20 mL scintillation vials. Samples were then wrapped with aluminium foil and kept frozen in the -20 ˚C freezer for further analysis.

Prior to HPLC analysis the samples were hydrolysed in order to obtain the total extracellular carbohydrates (TCHO), according to Myklestad et al. (1997). In brief, 4 mL of the sample and 0.4 mL of 1 M hydrochloric acid (HCl) were added to 5 mL glass ampoules. The ampoules were sealed and placed in a heat cabinet at 100 ˚C for 20 hours. After hydrolysis, 0.4 mL of 1 M NaOH was added in the samples to neutralise them. Hydrolysed samples were then kept in the dark and frozen until analysis.

4.3.4. Microphytobenthic biomass analysis

The MPB biomass at the three depths was also determined. The chlorophyll was extracted with 10 mL of methanol and measured on a field fluorometer (Turner Designs 10AU, Sunnyvale, CA, USA) using the acidification method (Holm-Hansen et al., 1965). The fluorometer was calibrated against a chlorophyll *a* standard (Sigma-Aldrich Chemical Co., St Louis, MO, USA).

4.3.5. Characterisation of carbohydrate fractions by High Performance Liquid Chromatography (HPLC)

Pre-treatment prior to HPLC; sugar standards of glucose, galactose, mannose, rhamnose, arabinose, and xylose were obtained from Sigma- Aldrich. The sugar composition in each sample was determined using High-Performance Liquid Chromatography (HPLC) with a NH2P-50 4E column (Shodex Asahipak, New York). The total retention time to process all sugars was 18 minutes. Peaks of neutral monosaccharides in the EPS were identified by comparison of retention time and mass spectral fragmentation patterns with monosaccharide standards (Fig. 4.3.2).

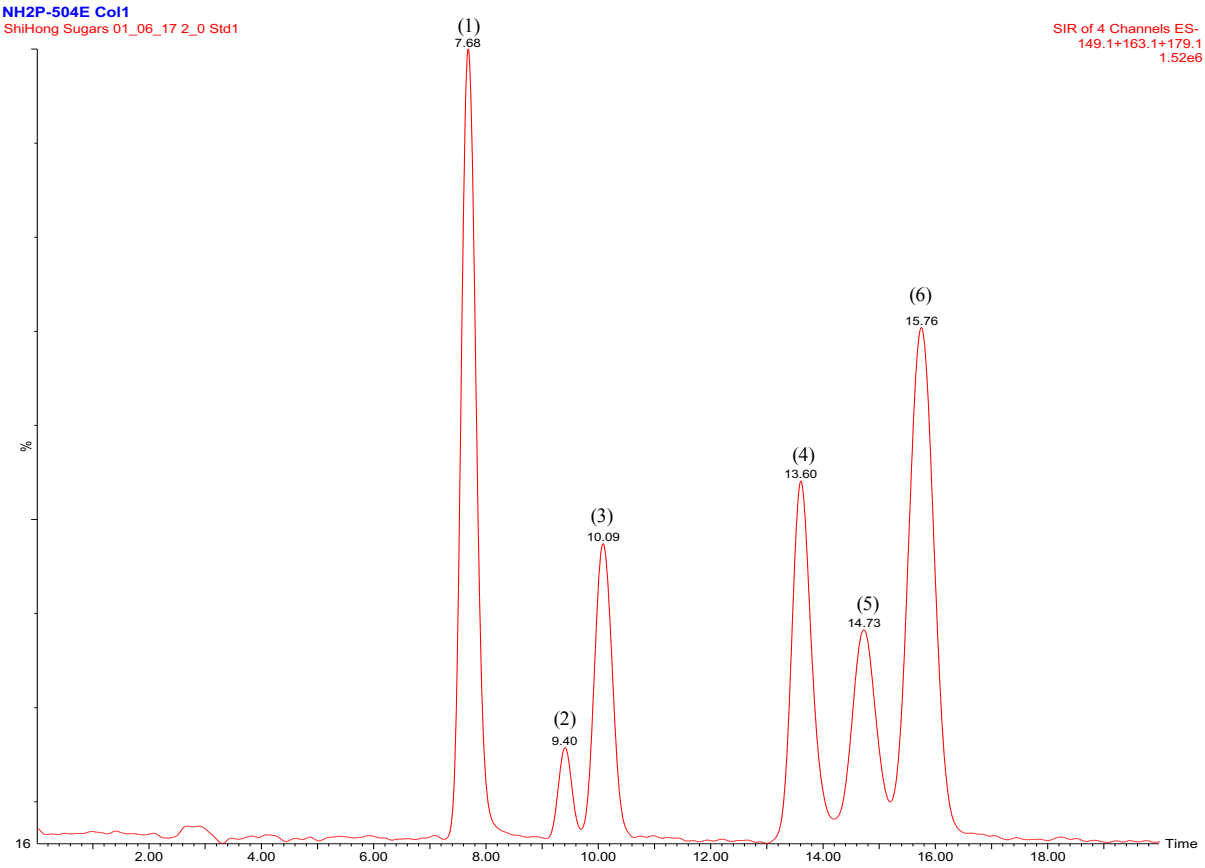


Figure 4.3.2. Gas chromatograms of six neutral sugars: (1) Rhamnose, (2) Arabinose, (3) Xylose, (4) Mannose, (5) Galactose, (6) Glucose, and their retention times (RTs) are 7.68, 9.40, 10.09, 13.60, 14.73, and 15.76, respectively.

4.3.6. Statistical analysis

Data were compiled in Microsoft Excel and statistical analyses were performed with the computing software, R (R-Core-Team, 2014). The relationship between the observed data and the environmental variables was investigated in order to acquire the most significant variables or combination of variables that had the most influences on the production of monosaccharides. Additionally, Analysis of Variance (ANOVA) was undertaken to study the most significant variation on monosaccharide production, temporally and spatially. A significance level of $P \leq 0.05$ was considered to be significant. Analysis of Covariate (ANCOVA) was also undertaken to determine the relationships between glucose and other environmental variables.

4.4. Results

4.4.1. Environmental variables

The sediment cores typically showed distinctive change in colouration below 4 mm (Fig. 4.4.1). While the surface sediment temperature naturally varied between seasons, (Table 3.1), there was little variation with depth (Table 4.4.1). Irradiance was also higher during spring and summer at both sampling sites (Table 4.4.1). Salinity though was lowest during winter at both sites (Table 4.4.1).

Figure 4.4.1. Typical sediment core showing change in coloration with depth.

Aligning with the sediment results in Chapter 2, the grain size analyses showed that the sediment at Penna Beach is sandy mud, with ≥ 70% of the sediments comprised of sediments with a grain size ≤ 180 μm, whilst at Kings Beach it is fine sand, with ≥ 70% of sediments comprised of grains ≤ 250 μm (Chapter 2: Fig. 2.4.1).

4.4.2. MPB species composition and distribution

A preliminary analysis of the species composition and distribution at each depth showed that there were no distinctive differences with depth. *Achnanthes reidensis*, *Achnanthes brevipes*, *Amphora coffeaeformis*, *Amphora laevissima*, *Cocconeis costata*, *Cocconeis peltoides*, *Lyrella david-mannii*, *Fallacia nyella*, *Navicula jeffreyae*, *Navicula rhynchocephala*, *Navicula salinarum*, *Navicula normaloides*, *Navicula menisculus*, *Nitzschia commutata*, *Gyrosigma distortum*, and *Gyrosigma turgida* were the dominant species at both sites (Chapter 2: Fig. 2.4.3), although these taxa were relatively more abundant at Penna Beach.

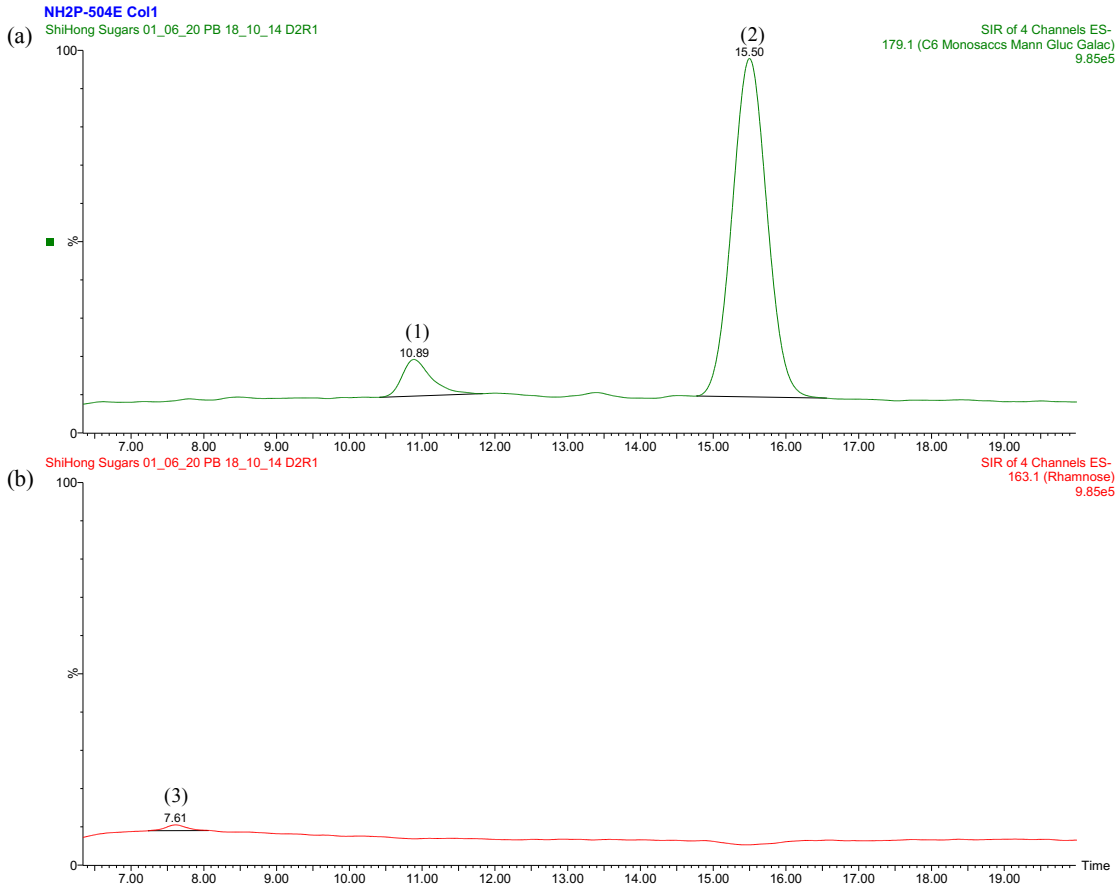
Table 4.4.1. Values of sediment temperature, irradiance, and salinity measured at both sampling sites during each season.

Sites	Seasons	Depths (mm)	Sediment Temperature (°C)	Ambient Irradiance (μmol photon $m^{-2}s^{-1}$)	Salinity
Kings Beach	Spring	1	22.8	2020	37.2
		3	22.7		
		5	22.6		
	Summer	1	19.9	2360	32.1
		3	20		
		5	20.1		
	Autumn	1	20	880	34
		3	20.1		
		5	20.2		
	Winter	1	9.2	600	30.9
		3	9.2		
		5	9.1		
Penna Beach	Spring	1	23.5	1470	36.6
		3	23.4		
		5	23.3		
	Summer	1	22.4	1310	36
		3	22.4		
		5	22.5		
	Autumn	1	18.5	625	34
		3	18.5		
		5	18.6		
	Winter	1	14.8	600	32.5
		3	14.7		
		5	14.6		

4.4.3. HPLC analysis

The HPLC analyses detected significant concentrations of glucose but relatively low concentrations of mannose, rhamnose, arabinose, xylose, and galactose (Fig. 4.4.2, Table 4.4.2). Additionally, glucose concentrations were also much higher than the total concentrations of all other carbohydrates.

There were significant differences in chl.*a*-normalised glucose between sampling sites, with significantly higher glucose concentration at Penna Beach, compared to Kings Beach (Table 4.4.3). The chl.*a*-normalised glucose varied significantly between seasons at Penna Beach, with spring having the highest concentrations. The chl.*a*-normalised glucose was relatively low at 3 mm and 5 mm depths during winter (Table 4.4.2 and 4.4.3, Fig. 4.4.3). In addition to glucose, mannose also varied significantly between seasons at Penna Beach, when both spring and autumn showed enhanced mannose production (p-values= 0.0427, F= 5.143, df= 3) (Table 4.4.2). Both chl.*a*-normalised glucose and chl.*a*-normalised mannose did not vary significantly with depth at Kings Beach (p-value= 0.874, F= 0.138, df= 2 and p-value= 0.422, F= 1.0, df= 2, respectively). There were only relatively low concentrations of glucose at Kings Beach and they did not vary significantly between seasons, or with depth. The highest glucose concentration at Kings Beach were found at a depth of 5 mm in winter (Table 4.4.2).



1590

1591 Figure 4.4.2. Chromatogram of HPLC-MS results of the extracted sediment sample. Sample
1592 was collected at 2 mm depth at Penna Beach during spring 2014, where (a) showed the results
1593 of xylose (1) and glucose (2) as targeted sugars and (b) showed the results of rhamnose (3) as
1594 the targeted sugar.

Table 4.4.2. Concentrations of each targeted monosaccharide, chl.*a* (mg chl.*a* m^{-2}) and phaeophytin (mg chl.*a* m^{-2}) pigments at both sampling sites and each season. The **bold** values are monosaccharides with a concentration greater than 0.01 mg/ mL. Note; With the exception of glucose other monosaccharides were at the limits of detection, values represent the chl.*a*-normalised quantity.

Sites	Seasons	Depth (mm)	Carbohydrate concentrations (mg/mL)						Pigments		
			Rhamnose	Arabinose	Xylose	Mannose	Galactose	Glucose	Chl.	Phaeo	Chl: Phaeo
Kings Beach	Spring	1	3.012×10^{-5}	3.012×10^{-5}	3.012×10^{-5}	3.012×10^{-5}	3.012×10^{-5}	**1.205×10^{-4}**	110.4 ± 3.1.	98.6 ± 2.9	1.120
		3	2.862×10^{-5}	2.862×10^{-5}	2.862×10^{-5}	2.862×10^{-5}	2.862×10^{-5}	**1.145×10^{-4}**	116.2 ± 7.0	103.7 ± 6.4	1.122
		5	1.718×10^{-4}	1.718×10^{-4}	1.718×10^{-4}	1.718×10^{-4}	1.718×10^{-4}	**5.154×10^{-4}**	19.4 ± 8.6	16.8 ± 7.5	1.111
	Summer	1	5.909×10^{-5}	5.909×10^{-5}	5.909×10^{-5}	5.909×10^{-5}	5.909×10^{-5}	5.909×10^{-5}	56.3 ± 6.7	51.1 ± 6.2	1.106
		3	7.793×10^{-5}	7.793×10^{-5}	7.793×10^{-5}	7.793×10^{-5}	7.793×10^{-5}	7.793×10^{-5}	42.7 ± 3.0	35.2 ± 1.6	1.207
		5	9.142×10^{-5}	9.142×10^{-5}	9.142×10^{-5}	9.142×10^{-5}	9.142×10^{-5}	9.142×10^{-5}	36.4 ± 2.9	30.9 ± 2.3	1.189
	Autumn	1	1.859×10^{-5}	1.859×10^{-5}	1.859×10^{-5}	1.859×10^{-5}	1.859×10^{-5}	**3.718×10^{-5}**	96.7 ± 24.4	73.1 ± 17.8	1.311
		3	2.447×10^{-5}	2.447×10^{-5}	2.447×10^{-5}	2.447×10^{-5}	2.447×10^{-5}	**4.895×10^{-5}**	90.5 ± 23.5	69.2 ± 18.5	1.317
		5	1.988×10^{-5}	1.988×10^{-5}	1.988×10^{-5}	1.988×10^{-5}	1.988×10^{-5}	1.988×10^{-5}	93.8 ± 25.4	72.4 ± 19.4	1.278
	Winter	1	3.438×10^{-5}	3.438×10^{-5}	3.438×10^{-5}	3.438×10^{-5}	3.438×10^{-5}	**6.876×10^{-5}**	178.9 ± 45.8	156.5 ± 35.4	1.135
		3	3.673×10^{-5}	3.673×10^{-5}	3.673×10^{-5}	3.673×10^{-5}	3.673×10^{-5}	**7.347×10^{-5}**	135.9 ± 38.6	109.6 ± 31.6	1.248
		5	3.545×10^{-5}	3.545×10^{-5}	3.545×10^{-5}	3.545×10^{-5}	3.545×10^{-5}	**3.084×10^{-3}**	167.3 ± 47.7	137.3 ± 40.3	1.234
Penna Beach	Spring	1	1.417×10^{-5}	1.417×10^{-5}	1.417×10^{-5}	4.251×10^{-5}	1.417×10^{-5}	**6.262×10^{-3}**	312.4 ± 24.9	244.9 ± 19.4	1.276
		3	1.089×10^{-5}	1.089×10^{-5}	1.089×10^{-5}	3.267×10^{-5}	1.089×10^{-5}	**4.214×10^{-3}**	315.0 ± 11.2	242.3 ± 7.8	1.301
		5	1.042×10^{-5}	1.042×10^{-5}	1.042×10^{-5}	3.127×10^{-5}	1.042×10^{-5}	**5.024×10^{-3}**	362.2 ± 14.9	274.3 ± 10.0	1.320
	Summer	1	9.588×10^{-6}	9.588×10^{-6}	9.588×10^{-6}	9.588×10^{-6}	9.588×10^{-6}	**2.397×10^{-4}**	346.8 ± 51.0	278.3 ± 33.7	1.235

	3	1.167×10^{-5}	1.167×10^{-5}	1.167×10^{-5}	1.167×10^{-5}	1.167×10^{-5}	**2.334×10^{-4}**	284.9 ± 27.8	239.1 ± 25.1	1.195
	5	1.003×10^{-5}	1.003×10^{-5}	1.003×10^{-5}	1.003×10^{-5}	1.003×10^{-5}	**2.408×10^{-4}**	331.5 ± 21.9	269.1 ± 19.4	1.235
	1	8.210×10^{-6}	8.210×10^{-6}	8.210×10^{-6}	1.642×10^{-5}	8.210×10^{-6}	**6.814×10^{-4}**	405.0 ± 30.6	335.2 ± 25.9	1.209
Autumn	3	8.516×10^{-6}	8.516×10^{-6}	8.516×10^{-6}	3.407×10^{-5}	8.516×10^{-6}	**1.925×10^{-3}**	390.4 ± 13.5	326.5 ± 9.8	1.195
	5	8.131×10^{-6}	8.131×10^{-6}	8.131×10^{-6}	8.131×10^{-6}	8.131×10^{-6}	**2.683×10^{-4}**	408.8 ± 29.6	335.2 ± 22.1	1.218
	1	1.065×10^{-5}	1.065×10^{-5}	1.065×10^{-5}	1.065×10^{-5}	1.065×10^{-5}	**3.406×10^{-4}**	234.7 ± 57.3	166.7 ± 38.2	1.118
Winter	3	1.056×10^{-5}	1.056×10^{-5}	1.056×10^{-5}	1.056×10^{-5}	1.056×10^{-5}	1.056×10^{-5}	305.4 ± 19.7	213.3 ± 13.3	1.431
	5	9.181×10^{-6}	9.181×10^{-6}	9.181×10^{-6}	9.181×10^{-6}	9.181×10^{-6}	9.181×10^{-6}	319.0 ± 22.2	227.6 ± 14.7	1.400

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Table 4.4.3. Nested ANOVA results of glucose production by MPB between sampling sites, and seasons and depths between sampling sites.

Parameters	df	Sum Sq.	Mean Sq.	F	P-value
Site	1	11.289	11.289	60.030	**8.381×10^{-7}**
Kings Beach					
Season	3	0.180	0.060	0.983	0.461
Depth	2	0.115	0.0571	0.939	0.442
Penna Beach					
Season	3	35.691	11.897	29.543	**5.442×10^{-4}**
Depth	2	0.111	0.0554	0.138	0.874

4.4.4. Microphytobenthic biomass analysis

The chl.*a* and phaeophytin concentrations at Penna Beach were significantly higher than those of Kings Beach (p-value= 4.295×10^{-11}, F= 325.849, df= 1 and p-value= 7.384×10^{-11}, F= 300.605, df= 1, respectively) (Table 4.4.2). Both chlorophyll and phaeophytin concentrations varied significantly with season at Penna Beach (p-value= 0.0169, F= 7.835, df= 3 and p-value= 0.00133, F= 21.35, df= 3, respectively), but not with depth (p-value= 0.308, F= 1.440, df= 2 and p-value= 0.309, F= 1.439, df=2, respectively) (Table 4.4.2). At Kings Beach both chlorophyll and phaeophytin concentrations also varied significantly with season (p-value= 0.0155, F= 8.137, df= 3 and p-value= 0.00239, F= 6.738, df= 3, respectively), but not with depths at this site (p-value= 0.377, F= 1.152, df= 2 and p-value= 0.336, F= 1.316, df= 2, respectively) (Table 4.4.2). The chl.*a*: phaeophytin ratio also varied significantly between sites (p-value= 0.0290, F= 5.921, df= 1). At Kings Beach this ratio also varied significantly with season (p-value= 0.00437, F= 13.610, df= 3) but it did not vary significantly with depth (p-value= 0.182, F= 2.294, df= 2). Chl.*a*: phaeophytin ratios did not vary significantly with

seasonal or with, depth at Penna Beach (p-value= 0.395, F= 1.173, df= 3 and p-value= 0.399, F= 1.074, df= 2, respectively).

There was weak correlation between glucose and chlorophyll concentrations, with glucose concentrations increasing with chlorophyll concentration (Fig. 4.4.3). This relationship was more evident at Penna Beach (Table 4.4.2).

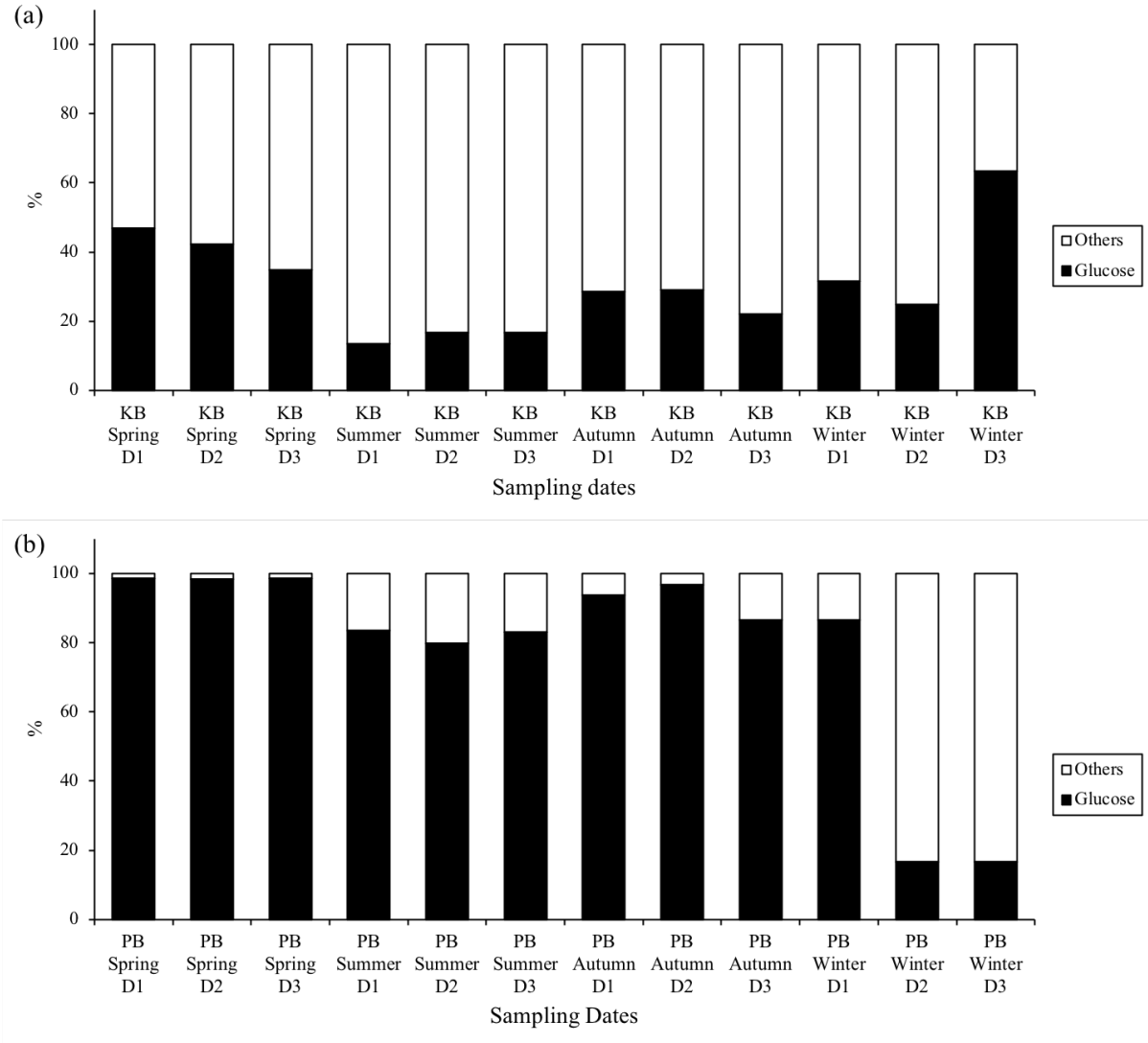

Figure 4.4.3. Bar charts showing the relationships between percentage of glucose (black) and percentage of total of other five sugars (white), at Kings Beach (a) and Penna Beach (b).

4.4.5. Relationship between glucose concentrations and environmental variables

ANCOVA results showed that glucose production correlated significantly with irradiance and salinity at Kings Beach (Table 4.4.4). This relationship may have been due to an elevated glucose concentration at one depth in particular at this site (i.e. 5 mm depth during winter)

(Table 4.4.2). However, there was no significant correlation between glucose and other environmental variables at Penna Beach (Table 4.4.4).

Table 4.4.4 ANCOVA results of microphytobenthic glucose production in response to different environmental variables at Kings Beach and Penna Beach.

	P-values	
Parameters	Kings Beach	Penna Beach
Depth T.	0.142	0.569
Light	**0.0481**	0.567
Precipitation	0.0641	0.994
Salinity	**0.0472**	0.620
Depth T.: light	0.0747	0.504
Depth T.: Precipitation	0.123	0.963
Depth T. Salinity	0.121	0.559

4.5. Discussion

This study investigated the depth profiles of extracellular colloidal monosaccharides in a southern temperate coastal region. HPLC results showed that not only was glucose the main sugar present in the extracted extracellular carbohydrate samples at both sampling sites, but also that it also had the highest concentration of all the targeted sugars. Previous studies have similarly shown that glucose usually comprises the largest proportion of carbohydrates produced *in situ* (Oakes et al., 2010, Taylor et al., 1999, Goto et al. 2001, and Pierre et al., 2012). It has also been found that the EPS fractions produced by *Cylindrotheca closterium* contained 82.5% and 22.9% of glucose in the non-attached (colloidal) and attached (bound) extracellular carbohydrates, respectively (Steele et al., 2014). There were significant differences in colloidal carbohydrate concentrations between sites. Extracellular carbohydrate concentrations have elsewhere been found to correlate strongly with chl.*a* concentrations (Bellinger et al., 2005, Underwood and Smith, 1998) and a similar pattern was observed in this study, particularly at Penna Beach which had higher concentrations of both chl.*a* and extracellular colloidal carbohydrate. Seasonal variations in glucose concentrations (surface) correlated strongly with both total carbohydrate and colloidal carbohydrate concentrations (Chapter 2, herein). Significant seasonal variations were observed in glucose concentrations at Penna Beach, with the highest concentrations occurring during spring and lowest during winter. However, there were no significant correlations between glucose concentrations and the measured environmental variables (Table 5.4).

The relatively high concentration of glucose measured at the sampling sites, particularly at Penna Beach, suggests 'photosynthetic overflow', under nutrient-limiting conditions, may have occurred. Maximum photosynthetic activity, for example, usually occurs in spring when environmental conditions are favorable, thus resulting in elevated extracellular carbohydrate production and nutrient limitation has been identified as the primary cause of glucose production as 'photosynthetic overflow' (de Brouwer and Stal, 2002; Marañón et al., 2004; Cook et al., 2004; Staats et al., 2000b). However, in this study, as nutrient concentrations at both sites were consistently high it is unlikely that the MPB was nutrient-stressed. Consequently, other factors need to be considered as contributing to these elevated glucose concentrations during spring, such as differences in the species composition. This will be researched in the upcoming coming to determine the species-specific glucose production in the MPB assemblages.

Bacteria consumption may be one of the fundamental causes of this increased glucose concentration. It has been shown that elevated glucose concentrations in sediment can be caused by the bacterial degradation of other carbohydrates (Goto et al., 2001, Pierre et al., 2012). Glucose is the preferred monosaccharide to be taken up by bacteria (Pierre et al., 2012), thus the extracellular carbohydrates tend to be broken down into this monosaccharide and being consumed by the bacteria (de Brouwer and Stal, 2001). Generally, most of the extracellular organic carbon produced by MPB and phytoplankton are labile organic compounds that are subsequently utilized by heterotrophic bacterial populations (Goto et al., 2001; Pierre et al., 2012; Miyatake et al., 2014; de Brouwer and Stal, 2001; McMinn and Lee, 2018). Goto et al. (1999) further showed that most of the extracellular carbohydrate fractions are utilized by bacteria, with more than 50% of added substrates mineralized within 24 hours. Glucose was generally high throughout the sampling period of this study. Therefore, extracellular carbohydrates may have been hydrolysed to glucose before being taken up by bacteria. Goto et al. (2001) found that β- and α-glucosidase activities were twice as high during low-tide at muddy sites than at sandy sites. In addition to bacterial utilization, the increased glucose concentrations may be due to the conversion of photosynthetic products into glucan by the MPB (Myklestad et al., 1989; Staats et al., 1999; Underwood et al., 1998).

The original aim of this study was to investigate the fate of the colloidal carbohydrate fractions throughout different depths of the sediment. However, this did not vary significantly between depths at either site (Table 3.2). Furthermore, not only did the total concentration not change, neither did the relative proportions of glucose, mannose, rhamnose, arabinose, xylose, and galactose. Also, neither the chl.*a* concentrations nor the chl.*a*: phaeophytin ratios varied significantly with depth. In this study (Chapter 3) the distribution of chl.*a* with depth has been used to provide an indication of active migration. The uniform distribution here thus indicates either an actively migrating MPB or significant burial by bioturbation. Chl *a*: phaeophytin ratios have typically been used to indicate community senescence (Yentch, 1965), with high values of phaeophytin reflecting the presence of dead or inactive cells. Here, the absence of change in this ratio with depth indicates not that cells are being buried but that they have remained active. The unchanging monosaccharide composition at different depths is thus an indication of active monosaccharide excretion with little subsequent modification by selective bacterial consumption. In other studies, concentrations of extracellular carbohydrates have been found to be highest at the surface, and decreased with increasing depths (Underwood and Smith, 1998; van Duyl et al., 1999; Yallop et al., 2000). Other studies have also found a more

pronounced depth distribution of MPB biomass in the sediment during low-tide, when MPB migrated down into the sediment to avoid photodamaging irradiance, or other unfavorable environmental conditions (Du et al., 2010; McLachlan et al., 2009; Jesus et al., 2006). The MPB communities in the current study showed little response to external environmental factors during low-tide and apparently were actively migrating down to 5 mm. Given how rapidly bacteria consume extracellular glucose, it is surprising that there was no greater difference between monosaccharide compositions at the surface and depth.

4.6. Conclusion

In conclusion, the HPLC data showed that glucose comprised a major proportion of the colloidal extracellular carbohydrates produced by MPB. Glucose concentrations varied significantly between sampling sites and between seasons. However, because nutrient concentrations were uniformly high the elevated glucose concentrations were most likely due to the hydrolysis or extracellular carbohydrates by bacteria and the production of glucan as the storage for dark survival. The glucose concentrations did not vary with depth, which may be indicating that they were undertaking vertical migration and bacterial consumption was not sufficient to significantly affect either the concentration or the composition of the monosaccharides. A more thoroughly investigation of extracellular carbohydrate throughout the emersion period at different depths is necessary.

Chapter 5: Microphytobenthic glucose production and consumption (Bacterial activities) in response to low nutrient and high irradiance in Tasmania, Australia, using modified glucose biosensors

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5.1. Abstract

Microphytobenthos (MPB) contribute approximately half the total primary production in coastal regions. MPB produce copious amount of extracellular carbohydrates as an adaptive strategy in highly dynamic tidal environments. Glucose comprises a significant fraction of the extracellular compounds excreted by MPB, and this is attributed to photosynthetic overflow.

Variable nutrient supply did not have significant impact on MPB with respect to glucose production for both single-species and mixed cultures. Photosynthetic performance (F_v/F_m and $rETR_{max}$) was influenced by nutrient concentration; higher F_v/F_m values were observed in nutrient-limited conditions. In mixed-species cultures, the bacterial consumption of algal-derived glucose was significantly reduced in the presence of antibiotics. In general, glucose production was higher in single-species cultures.

This study validates the use of glucose biosensors for measuring real-time changes in microphytobenthic carbohydrate production. Nutrient concentrations and subsequent glucose overflow by MPB appear to be species-specific, as does the rate of glucose consumption by bacteria. The use of antibiotics was less effective in mixed-species cultures and glucose production was generally lower, presumably because of increased inter-specific competition.

5.2. Introduction

Microphytobenthos (MPB) are benthic microalgae that are fundamentally important to ecosystem dynamics in coastal regions (Blanchard et al., 2000, Cahoon et al., 1999, Underwood and Provot, 2000, Underwood and Kromkamp, 1999). Most microphytobenthic communities are concentrated in the photic zone of the intertidal; areas that exhibit high physicochemical variability (Cartaxana et al., 2013; Du et al., 2010; Lee and McMinn, 2013). MPB are required to adapt to both spatial and temporal change within near-surface sediments at scales ranging from hourly through to seasonal. Behavioural adaptive strategies include vertical migration, which is orientation to a specific depth within the sediment to prevent damage to the cells' photosynthetic apparatus (Jordan *et al.*, 2008; *Perkins et al.*, 2010). A complimentary strategy is photoacclimation, which is process whereby cells employ thermal dissipation to offset harmful excess energy (Lavaud *et al.*, 2004; Chevalier *et al.,* 2010). This is achieved using non-photochemical quenching (NPQ), which is induced by the diadinoxanthin (Dd) cycle and conversion of Dd to diatoxanthin (Dt) (Jesus *et al.*, 2009).

Previous studies have shown that the adaptive responses exhibited by MPB are correlated with the production of the extracellular carbohydrates (Smith and Underwood, 2000; Staats et al., 2000a; Underwood et al., 2004; Underwood and Smith, 1998). Microbially-produced extracellular polymeric substances (EPS) are thought to protect against rapid environmental changes and increase resistance of cells to desiccation, heavy metals and low organic carbon supply (Decho and Lopez, 1993).

The primary driver of EPS production is light, but other contributing variables include: time of exposure (Underwood and Paterson, 1993), algal growth stage (Staats *et al.*, 1999; Smith and Underwood, 2000), tidal cycles, and mudflat morphology (Taylor and Paterson, 1998; Blanchard *et al.*, 2000). Epipelic diatoms are the dominant group of MPB in the intertidal zone, and they produce copious extracellular carbohydrates (Blanchard et al., 2000, Underwood et al., 1995). In fact, it appears that the amount of secreted polysaccharides can exceeded that required for migration within sediment. This "overflow metabolism" is hypothesised to be a response to localised nutrient availability. Nutrient-replete cells have been shown to produce a complex EPS comprising rhamnose, fucose, xylose, mannose, galactose, glucose, and uronic acids. In contrast, nutrient-limited cells produced EPS that did not contain rhamnose, fucose, or xylose (Underwood et al., 2004). Glucose is one of the major components of extracellular carbohydrate produced by MPB (Blanchard et al., 2000; de Brouwer and Stal, 2001; de

Brouwer and Stal, 2002; Stal, 2003; Yallop et al., 1994). Previous studies suggest that > 50% of total extracellular carbohydrates comprise glucose (Smith and Underwood, 1998; de Brouwer and Stal, 2001; de Brouwer and Stal, 2002; Staats et al., 2000a; Pierre et al., 2014). Bacterial consumption is another important process that influences the composition of extracellular carbohydrates in microbial ecosystem, as the extracellular carbohydrate can be rapidly broken and taken up by the bacteria (van Duyl *et al.*, 1999; Oake and Eyre, 2014). Excreted carbohydrates are rapidly degraded by heterotrophic consumers such as bacteria (de Brouwer and Stal, 2001) through a process which utilises exo-enzymes to hydrolyse carbohydrates into smaller molecules (Weiss et al., 1991).

Because glucose represents a significant fraction of the microphytobenthic extracellular carbohydrate, it is important to qualify how environmental drivers influence production. In this lab-based study, a modified glucose biosensor (Pinnacle Technology, Lawrence, Kansas, USA) is used to provide real-time *in vitro* measurements of changes in the concentration of glucose secreted by MPB. Specifically, this study aims to (1) investigate glucose production in three epipelic diatom species, (2) determine the glucose production of artificial microphytobenthic biofilms in response to abiotic stimuli, and (3) determine the glucose production and consumption in the mixed microphytobenthic communities from natural sediments.

5.3. Materials and methods

5.3.1. Organisms

Three epipelic diatoms, *Halamphora coffeaefirmis*, *Navicula menisculus*, and *Nitzschia longissima* were isolated from samples collected at Penna Beach, Tasmania (42°47'06"S, 147°31'13"E) (Fig. 5.3.1). In addition to sediment collected for the single species isolations, sediment cores were also collected from the same sampling site using a 45 mm diameter sediment corer.

Figure 5.3.1. Penna Beach, sampling site for culture collections.

5.3.2. Culture conditions

To establish single-species cultures, species of interest were isolated using standard isolation techniques. This included repeated isolation and subculturing prior to cells being transferred to 50 mL culturing flasks containing L/1 media (CSIRO, Hobart, Tasmania). Species identification was confirmed using a Hitachi SU-70 Analytical Field Emission Scanning Electron Microscope (FESEM) (Hitachi, Naka Division, Ibaraki Prefecture). All cultures were incubated at 25°C with a 12/12 light (100 µmol m^{-2} s^{-1}) -dark regime. Subculturing was conducted fortnightly, to ensure late-logarithmic growth phase development was reached, by transferring 1 mL of parent cultures to a fresh batch of L/1 medium. Prior to experimentation, the media was replaced on a weekly basis for four weeks to make sure that each flask had enough nutrients for the cultures to retain exponential growth. For biofilm development,

sediment was collected from the sampling site and sterilised using an autoclave machine (Atherton TXX6 steriliser; Northcote, VIC, Australia). 15 g of the sterilised sediments was then inserted as the substratum at the bottom of each culture flask. Excess media was carefully removed from the culture flasks prior to experimentation using a sterile plastic syringe, until only a thin liquid film remained over the biofilm.

For the natural sediment, the top 2 mm of sediment was sectioned and incubated in L/1 media prior to the experiments. The cultures were then grown in similar conditions to those of the monocultures.

5.3.3. Photosynthetic overflow analysis

A modified Pinnacle Technology (USA) biosensor was used to measure the real-time changes in glucose production. This sensor uses the glucose oxidase enzyme to reduce the glucose molecules and produce hydrogen peroxide as a by-product. The hydrogen peroxide is reduced on a platinum electrode to produce electrons that are then detected on a Ag/AgCL reference electrode (Fig. 5.3.2).

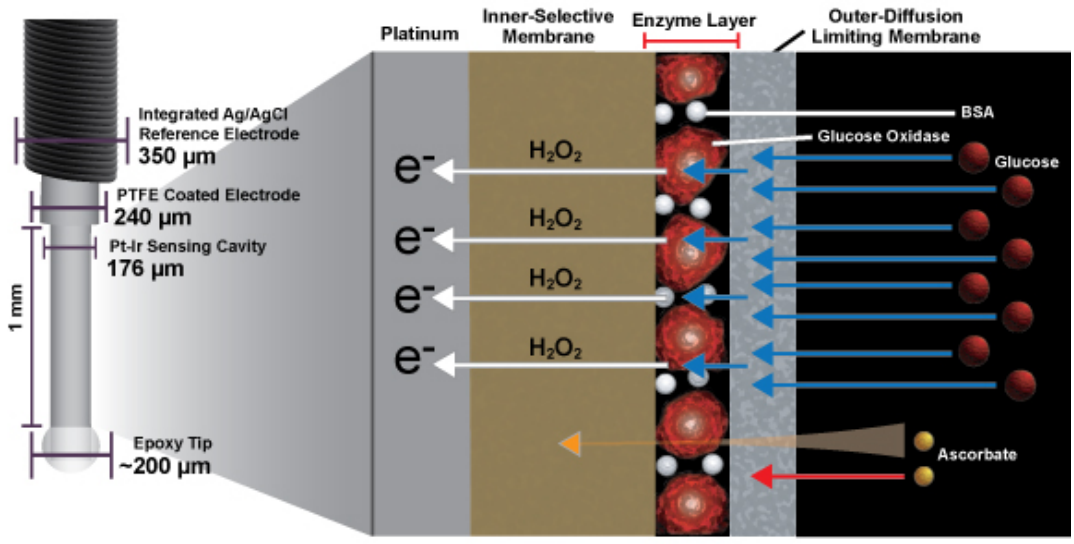


Figure 5.3.2. Diagram showing that how does the biosensor operate. Reprinted from Glucose biosensors, by Pinnacle Technology Inc, 2015, retrieved from (http://www.pinnaclet.com/glucose.html). Copyright (2015) by Pinnacle Technology.

This biosensor was originally designed to measure the glucose concentration in the brains of rodents for the pharmaceutical industry. Hence, modification by the company was required for the biosensors to be able to measure microbial glucose production. A pilot study was carried

out in order to assess the reliability of the biosensor. Calibration against a standard curve was performed for each biosensor prior to use. A D-glucose standard (Sigma Aldrich, USA) was made by diluting a 10 mg L^{-1} stock solution of glucose to concentrations of 0.25, 0.5, 1.0 and 2.0 mg L^{-1}, equivalent to 8.33, 16.67, 33.33 and 66.67 µmol C per litre. Standard curves were used to calculate the glucose concentration of each culture during the experiments.

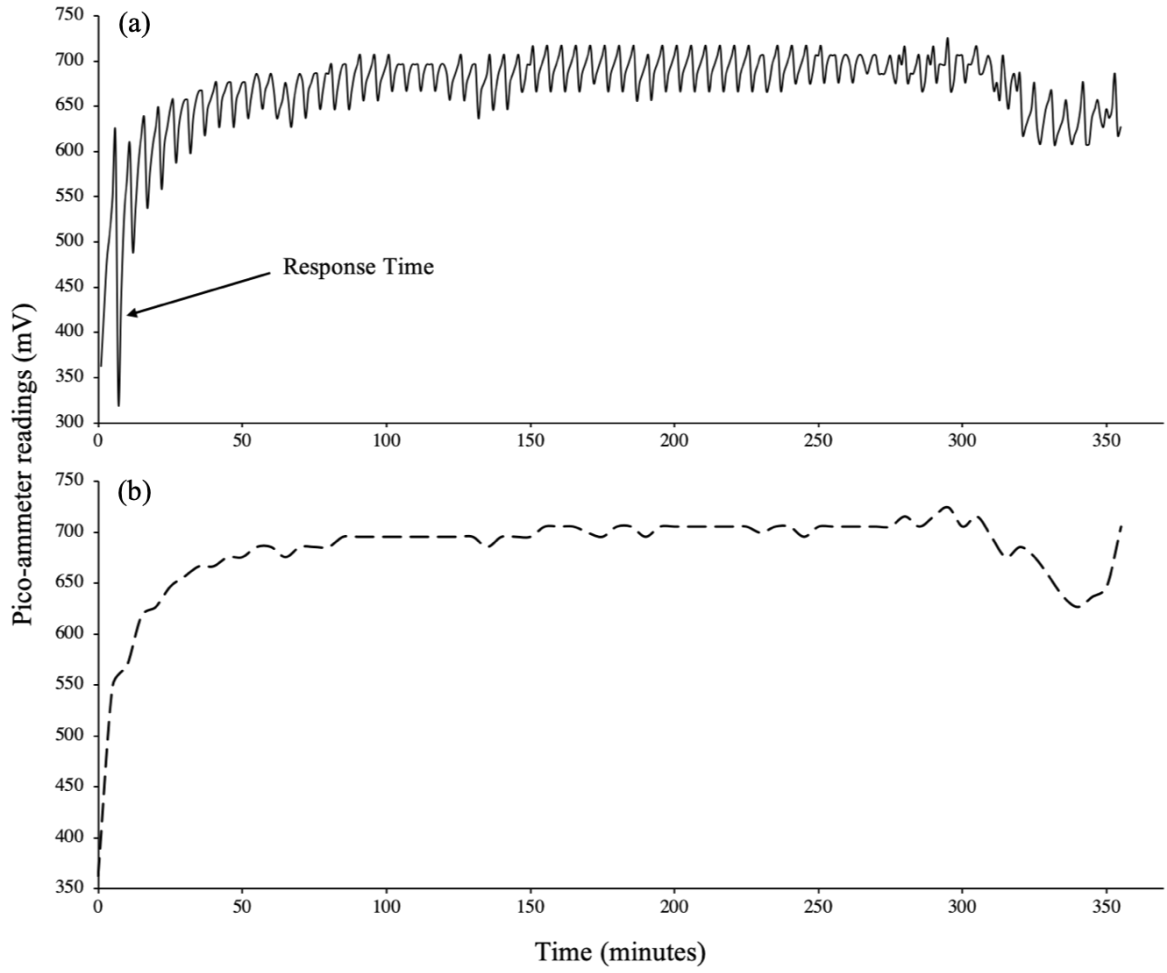


Figure 5.3.3. Glucose production by *Navicula minisculus* during a pilot study. Data logged at 1-minute intervals (a) and averaged over of 5-minutes intervals (b).

Although the biosensors had a relatively rapid response time, they were highly sensitive to movement in the surrounding environment such as knocking on the bench and vibrations from doors closing etc (Fig. 5.3.3). In order to minimize the effect of the exterior interference, the data was logged at a fast rate (every 1 minute) and the averaged over a longer time interval (Every 5 minutes) (Fig. 5.3.3).

5.3.4. Experiment designs

5.3.4.1. Treatments

Overhead light was provided by sports lights (Osram Vialox NAV (SON)-E, 400 Watt) at the following levels: 67, 140, 300, and 933 μmol photons $m^{-2}s^{-1}$. The irradiance was adjusted using neutral density filters comprised of a UV blocking film (UV Light Technology Limited, Birmingham, United Kingdom). The irradiance was progressively stepped up from the dark through the four light levels at hourly intervals. The light was switched off for an hour after the highest light intensity. Sediment temperature of samples was controlled at 25 °C by using a temperature probe (HI 766C) (K-type thermocouple thermometer, HI935005, Hanna Instruments, Woonsocket, Rhode Island, USA).

Prior to the commencement of each experiment, the cultures were exposed to different growth conditions. For the nutrient experiments, the control cultures were grown under nutrient replete conditions, this was ensured by replacing the media weekly. Nutrient deficiency was achieved by starving the cultures for 20 days prior to the experiment. To verify that the medium was depleted, 10 mL of the medium was collected for nutrient analysis and measured with a nutrient multianalyser (QuickChem 8500 Series, Lachat Instruments, Hach Company, Loveland, United States).

In addition to the nutrient-limitation experiments, the consumption of glucose by bacteria was also studied. This was achieved by manipulating the bacteria in the selected cultures. The control cultures were exposed to the surrounding environment with the lid of the flask lids being removed for a day, to permit bacterial growth before the commencement of the experiments. Additional replicates of the same cultures were added to the natural sediments and grown for a week, with the addition of 1 mL of 100 μg/L of Antibiotic Antimycotic Solution (Sigma Aldrich, Australia). This removed bacteria from the cultures. The rate of bacterial consumption was then determined by calculating the rate of changes in glucose concentration after the light was turned off.

5.3.4.2. Chlorophyll fluorescence measurements

Chlorophyll fluorescence was measured using a high-resolution Pulse Amplitude Modulated (PAM) fluorometer (Diving-PAM; Walz, Effeltrich, Germany) and Rapid Light Curves (RLCs) were obtained with a light treatment consisting of a saturating pulse of light followed by eight

consecutive 10 s intervals of increasing actinic light of 0, 54, 76, 117, 160, 250, 371, 557, and 939 μmol photon $m^{-2}s^{-1}$ and photosynthetic efficiency (F_v/F_m) and maximum relative electron transport rate ($rETR_{max}$) were acquired following Ralph and Gademann (2005). The fibre optic cable of a Diving-PAM was attached to a micromanipulator in order to standardise the distance from the sediment surface. Prior to the incubations, the flasks were wrapped with aluminium foil in order to dark-adapt the cultures for 30 minutes.

5.3.4.3. Biomass measurements

The biomass of 5g of sediment containing the cultures was estimated by extracting the chlorophyll-*a* in 10 mL of methanol overnight at 4°C in the dark. Fluorescence was measured on a Turner Designs 10AU fluorometer (Sunnyvale, CA, USA) using the acidification method of Holm-Hansen *et al.* (1965).

5.3.4.4. Extracellular carbohydrate measurements

The glucose biosensor was mounted onto a metal rod to and fixed onto a micromanipulator alongside the diving-PAM cable. The glucose production of each culture was measured by inserting the biosensor into the culture, down to 1 mm above the sediment surface using the micromanipulator. The biosensor was connected to a picoammeter (PA2000, Unisense, Denmark). The readings were converted into mg of glucose per litre (mg glucose L^{-1}) using standard curves. The rate of production was then normalized to chl.*a* concentrations (mg glucose [mg chl.*a*]$^{-1}hr^{-1}$).

The standard curve for D-glucose was made by diluting a 10 mg L^{-1} stock solution, to 1, 0.1, 0.01, 0.001, $1x10^{-4}$, $1x\ 10^{-6}$ mg L^{-1}.

5.3.5. Statistical analysis

Data were compiled in Microsoft Excel and statistical analyses were performed with computing software, R (R Core-Team, 2014). The rate of glucose production was plotted against different variables in order to study its relationship to all different variables. Analysis of variance (ANOVA) was used to test for variation between treatments with respect to both nutrient level and bacterial consumption.

5.4. Results

In these experiments, the MPB cultures were exposed to incremental increases in irradiance to determine the effect of light on *in vitro* glucose production. The glucose production commenced once the culture was exposed to light. Additionally, the rate of production increased with higher light intensities (Figure 3.1) and this was similar for all single-species incubations, as well as the natural MPB communities.

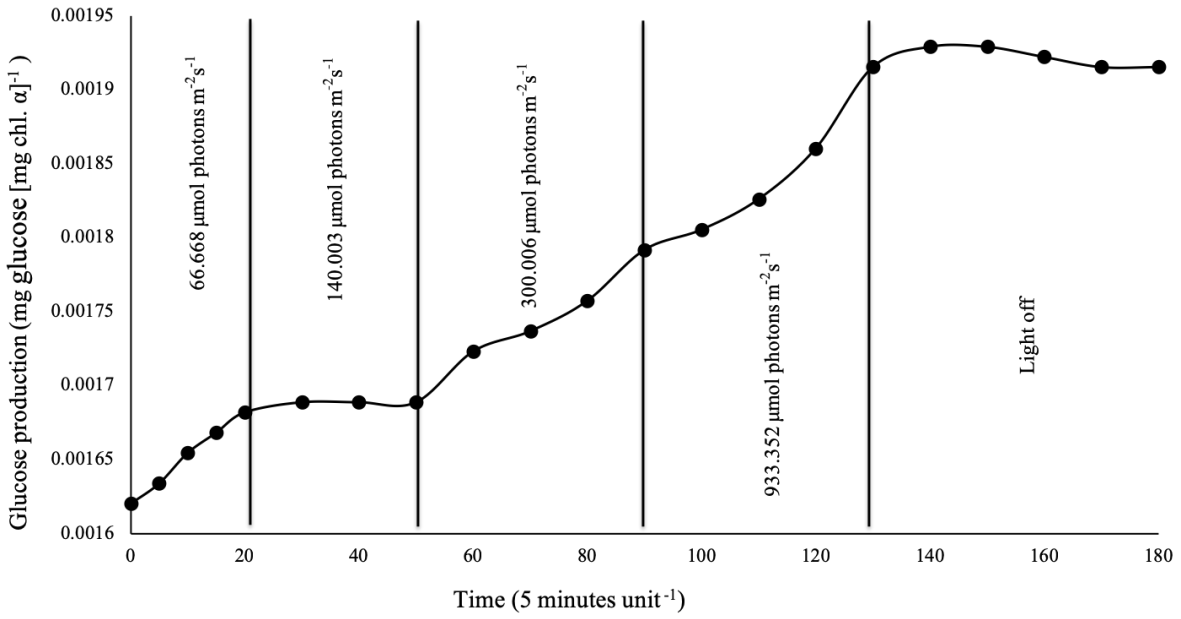


Figure 5.4.1. Glucose concentration of *H. coffeaefirmis* (mg glucose [mg chl.*a*]$^{-1}$) over time.

5.4.1. Nutrient Treatments

5.4.1.1. H. coffeaefirmis

The highest rate of glucose production by *H. coffeaefirmis* under nutrient-replete and nutrient–limited conditions was 0.0455 mg glucose [mg chl.*a*] $^{-1}$ hr^{-1} and 0.454 mg glucose [mg chl.*a*] $^{-1}$ hr^{-1}, respectively. The lowest rate of glucose production by *H. coffeaefirmis* under nutrient-replete and nutrient–limited conditions was 0.00758 glucose [mg chl.*a*] $^{-1}$ hr^{-1} and 0.0252 glucose [mg chl.*a*] $^{-1}$ hr^{-1}, respectively. However, there was significant difference in production rates between the two treatments (p-value= 0.229, F= 1.789, df= 1). For the photosynthetic assessment, there was a significant treatment effect; both F_vF_m and $rETR_{max}$ values of nutrient-saturated were higher than those of nutrient-limited cultures (p-value= 0.00285, F= 23.531, df= 1, p-value= 0.0473, F= 6.193, df= 1, respectively) (Fig. 5.4.2). There were significant

correlations between the rate of glucose production and light, F_v/F_m, and $rETR_{max}$ in the nutrient-saturated experiments. These parameters essentially declined with increasing irradiance (Fig. 5.4.2 a-c). In the nutrient-limited experiments, an opposite trend was observed with a positive correlation between glucose production with increased irradiance (Fig 5.4.2 d). However, the rate of production decreased with increasing F_v/F_m (Fig. 5.4.2 e).

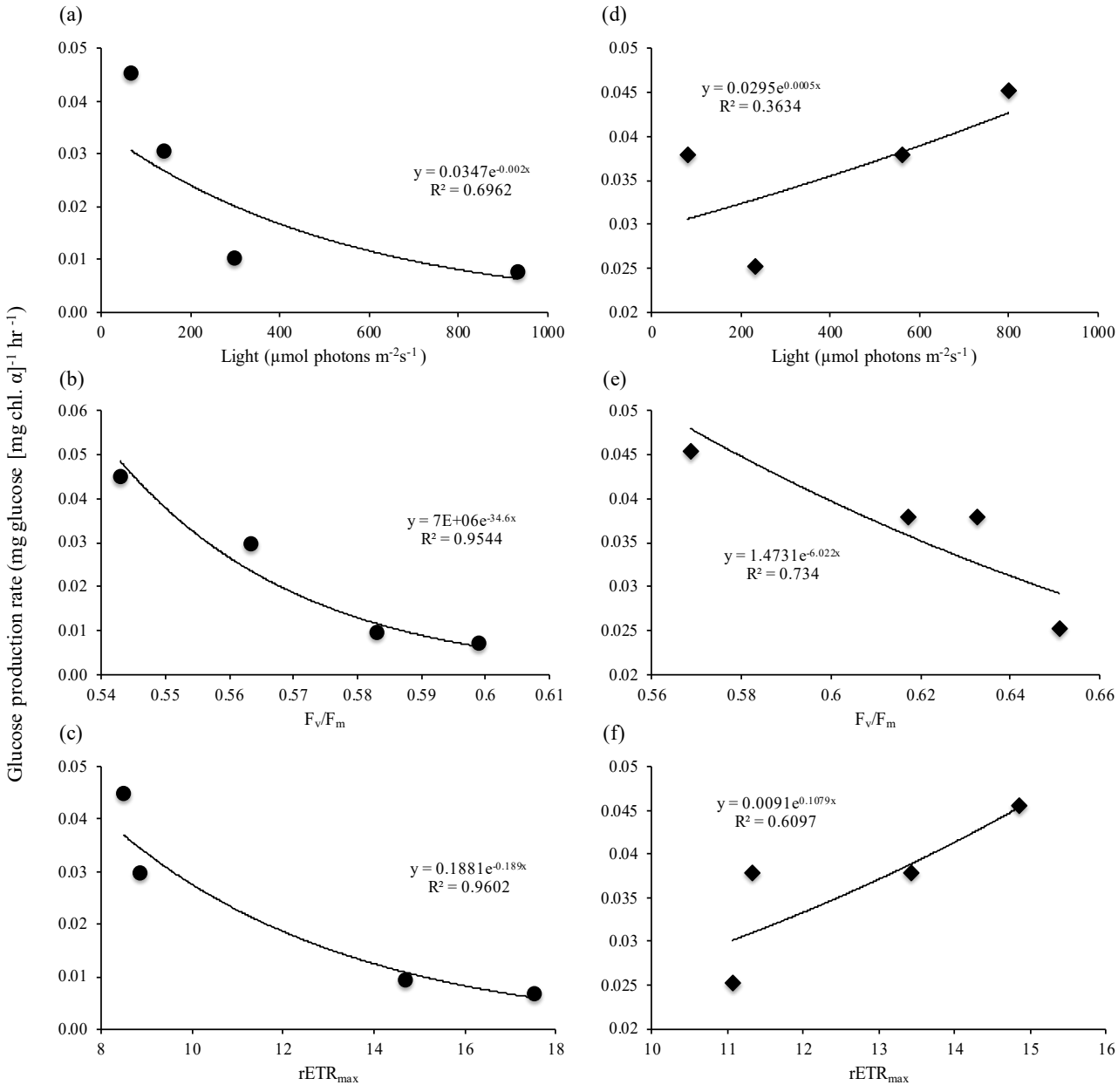


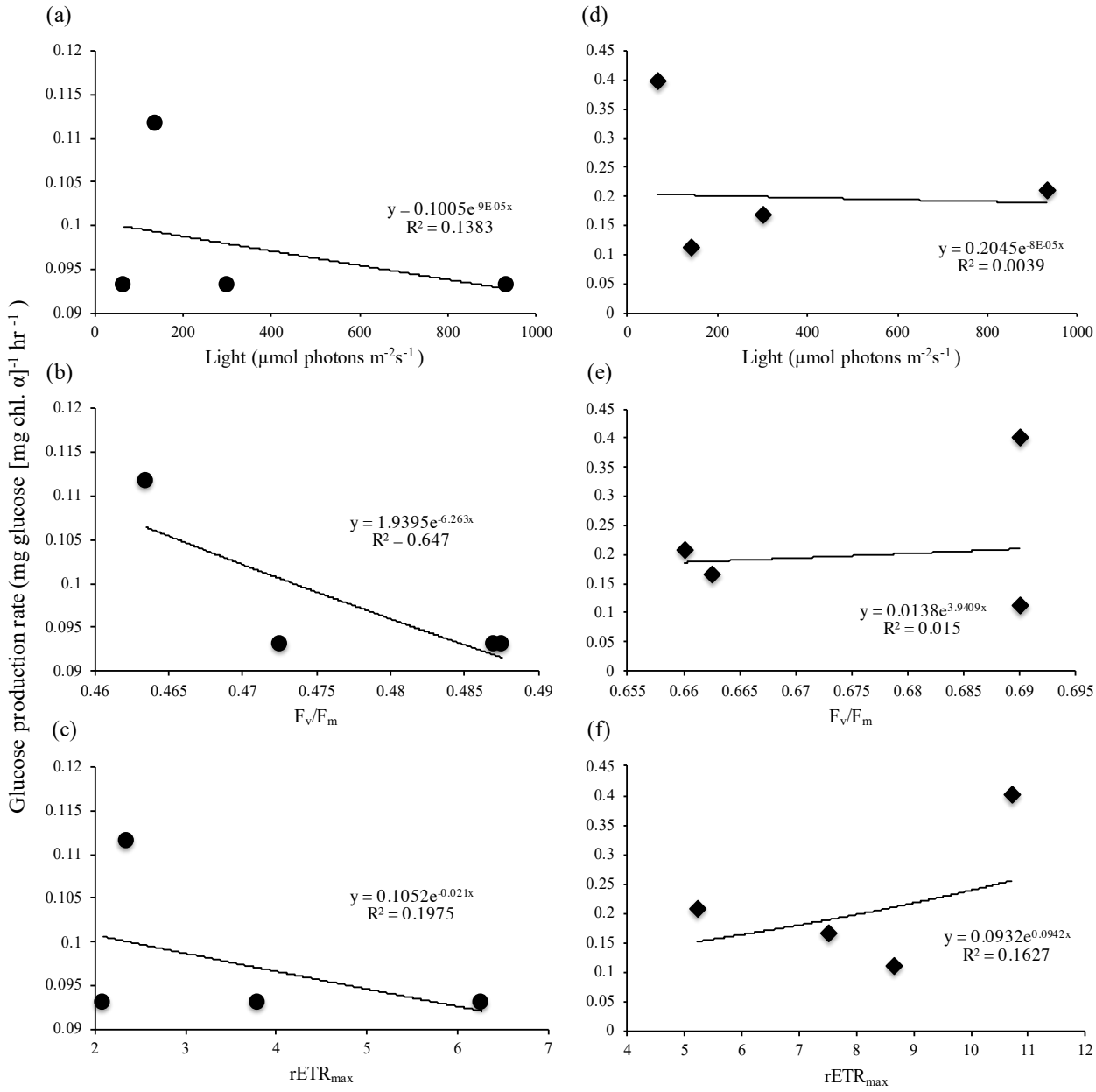
Figure 5.4.2. The relationship between the rate of glucose production and light, F_v/F_m, and $rETR_{max}$ by *A. coffeaeformis* in nutrient saturated [(a), (b), (c)] and nutrient limited [(d), (e), (f)] media.

5.4.1.2. N. menisculus

The highest rate of glucose production by *N. menisculus* observed under nutrient-replete and nutrient–limited conditions was 0.112 mg glucose [mg chl.*a*] $^{-1}$ hr^{-1} and 0.400 mg glucose [mg chl.*a*] $^{-1}$ hr^{-1}, respectively. The lowest rate of glucose production by *N. menisculus* under nutrient-replete and nutrient–limited conditions was 0.0931 glucose [mg chl.*a*] $^{-1}$ hr^{-1} and 0.111 glucose [mg chl.*a*] $^{-1}$ hr^{-1}, respectively. There was no significant difference in the rate of glucose production between treatments (p-value= 0.0963, F= 3.883, df= 1). However, nutrient treatment had a significant effect on the photosynthetic performance of *N. menisculus*. Both F_vF_m and $rETR_{max}$ values of nutrient-replete cultures were higher than those of nutrient-limited cultures (p-value= 1.19x10^{-6}, F= 379.13, df= 1, p-value= 0.0260, F= 8.636, df= 1, respectively) (Fig. 5.4.3). There were weak correlations between the rate of glucose production and light, F_v/F_m, and $rETR_{max}$ in the nutrient-replete cultures, although this decreased with increasing irradiance, except at the light intensity of 140.003 µmol photons m^{-2}s^{-1} (Fig. 5.4.3 a-c). In the nutrient-limited cultures, the opposite trend was apparent; glucose production increased with increased irradiance (Fig 5.4.3 d). However, glucose production decreased with increasing F_v/F_m and $rETR_{max}$ (Figure 5.4.3 e-f).

5.4.1.3. N. longissima

The highest rate of glucose production in *N. longissima* under nutrient-replete and nutrient–limited conditions was 0.241 mg glucose [mg chl.*a*] $^{-1}$ hr^{-1} and 0.150 mg glucose [mg chl. *a*] $^{-1}$ hr^{-1}, respectively. The lowest rate of glucose production by *N. longissima* under nutrient-replete and nutrient–limited conditions was 0.121 glucose [mg chl.*a*] $^{-1}$ hr^{-1} and 0.100 glucose [mg chl.*a*] $^{-1}$ hr^{-1}, respectively. There was no significant difference between the two treatments with respect to production rate (p-value= 0.195, F= 2.127, df= 1). Nutrient availability had a significant effect in the photosynthetic performance of *N. longissimi*; F_vF_m values of nutrient-replete cutlures were higher than those of nutrient-limited cultures (p-value= 0.0276, F= 8.363, df= 1) (Fig. 5.4.4). The parameter $rETR_{max}$ was not significantly different (p-value= 0.260, F= 1.544, df= 1). Weak correlations were observed between the rate of glucose production and light, F_v/F_m, and $rETR_{max}$ in the nutrient-replete experiments (Fig. 5.4.4 a-c). In the nutrient-limited experiments, the rate of glucose production of *N. longissima* correlated positively with light intensities, where it increased with increasing light intensities, but the rate of glucose production decreased with increasing photosynthetic performance, for both F_v/F_m and $rETR_{max}$ (Fig. 5.4.4 d-f).



1960

1961 Figure 5.4.3. The relationship between the rate of glucose production and light, F_v/F_m, and
1962 $rETR_{max}$ by *N. menisculus* in nutrient saturated [(a), (b), (c)] and nutrient limited [(d), (e), (f)]
1963 media.

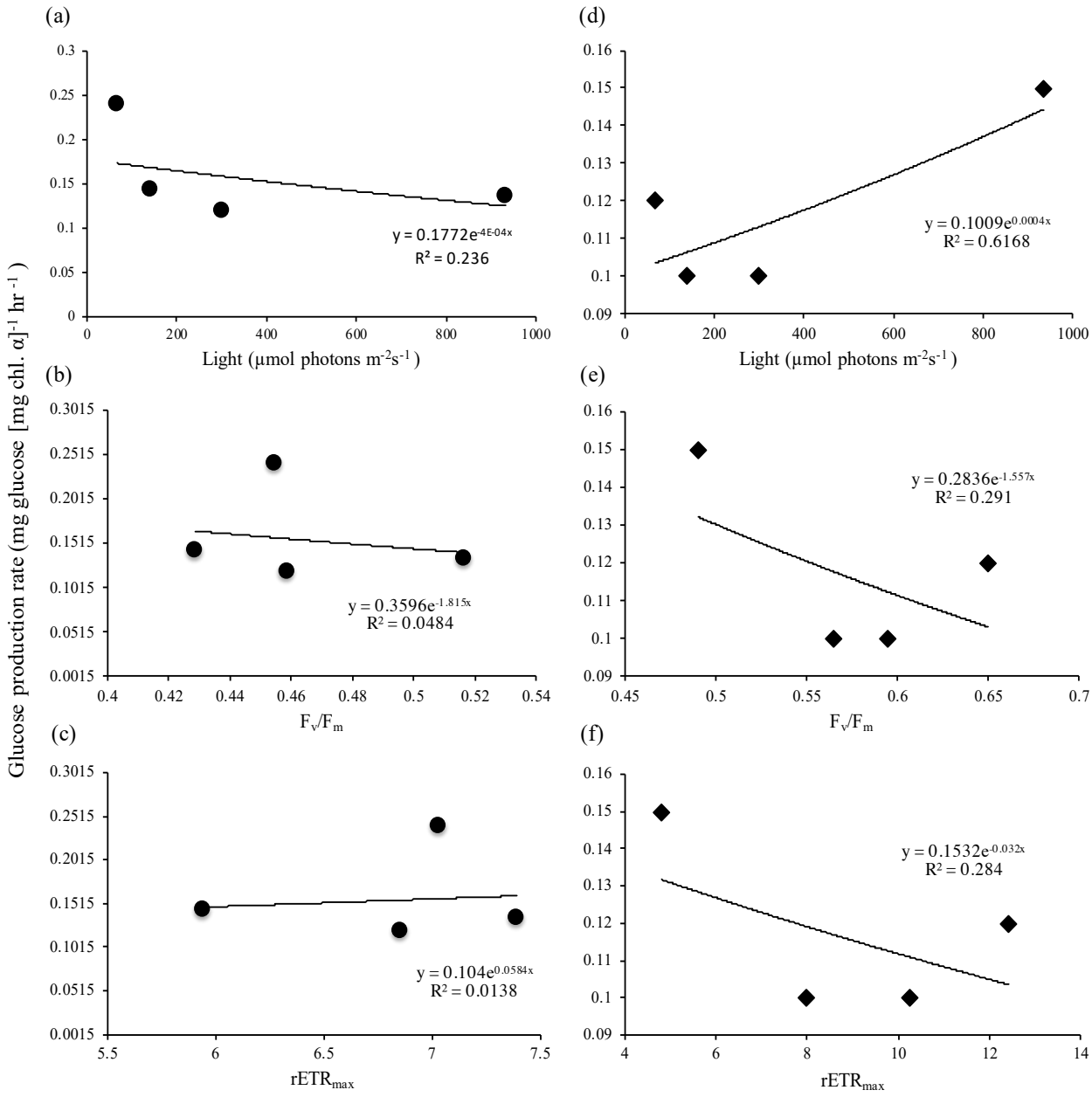


Figure 5.4.4. The relationship between the rate of glucose production and light, F_v/F_m, and $rETR_{max}$ by *N. longissima* in nutrient saturated [(a), (b), (c)] and nutrient limited [(d), (e), (f)] media.

5.4.2. Bacterial glucose consumption

Cultures of the same three species were incubated for 4 hours, but instead of limiting the nutrient levels, antibiotics were added prior to incubation. In general, glucose consumption by bacterial was minimal for the cultures spiked with antibiotics. This trend was inferred in the final 40 minutes of the incubation, when the light was switched off (Fig 5.4.5). In *H.*

coffeaefirmis (Fig. 5.4.6) and *N. menisculus* (Fig. 5.4.7) cultures, the rate of production was higher in those grown with antibiotics than those without antibiotics, However, *N. longissima* showed only a slight and insignificant increase in the rate of production when antibiotics were added (Figure 3.8).

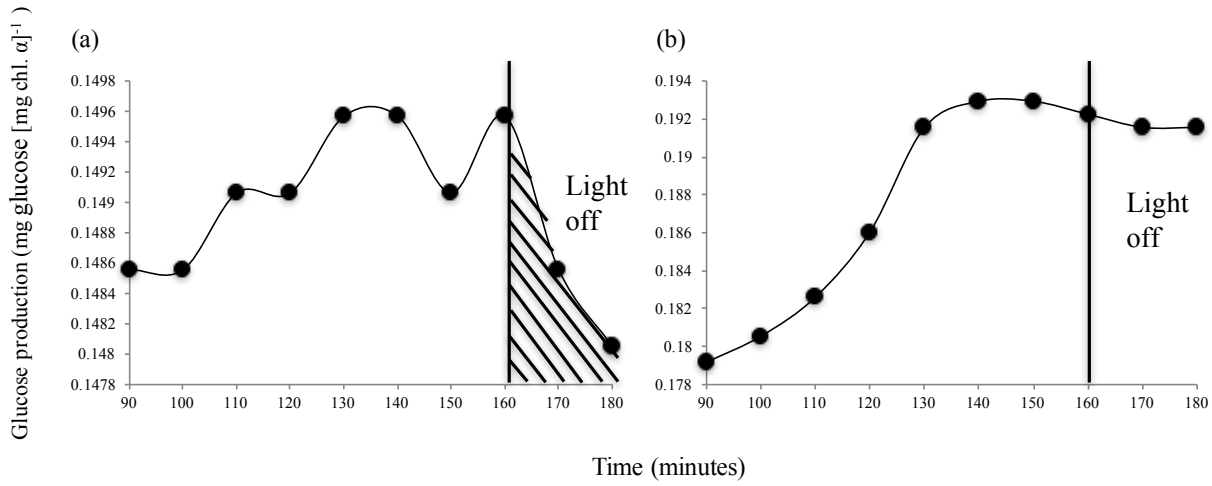


Figure 5.4.5. The glucose production of *H. coffeaefirmis* in the final 90 minutes of the incubation, where (a) is the control, whilst (b) is the culture that was added with antibiotics.

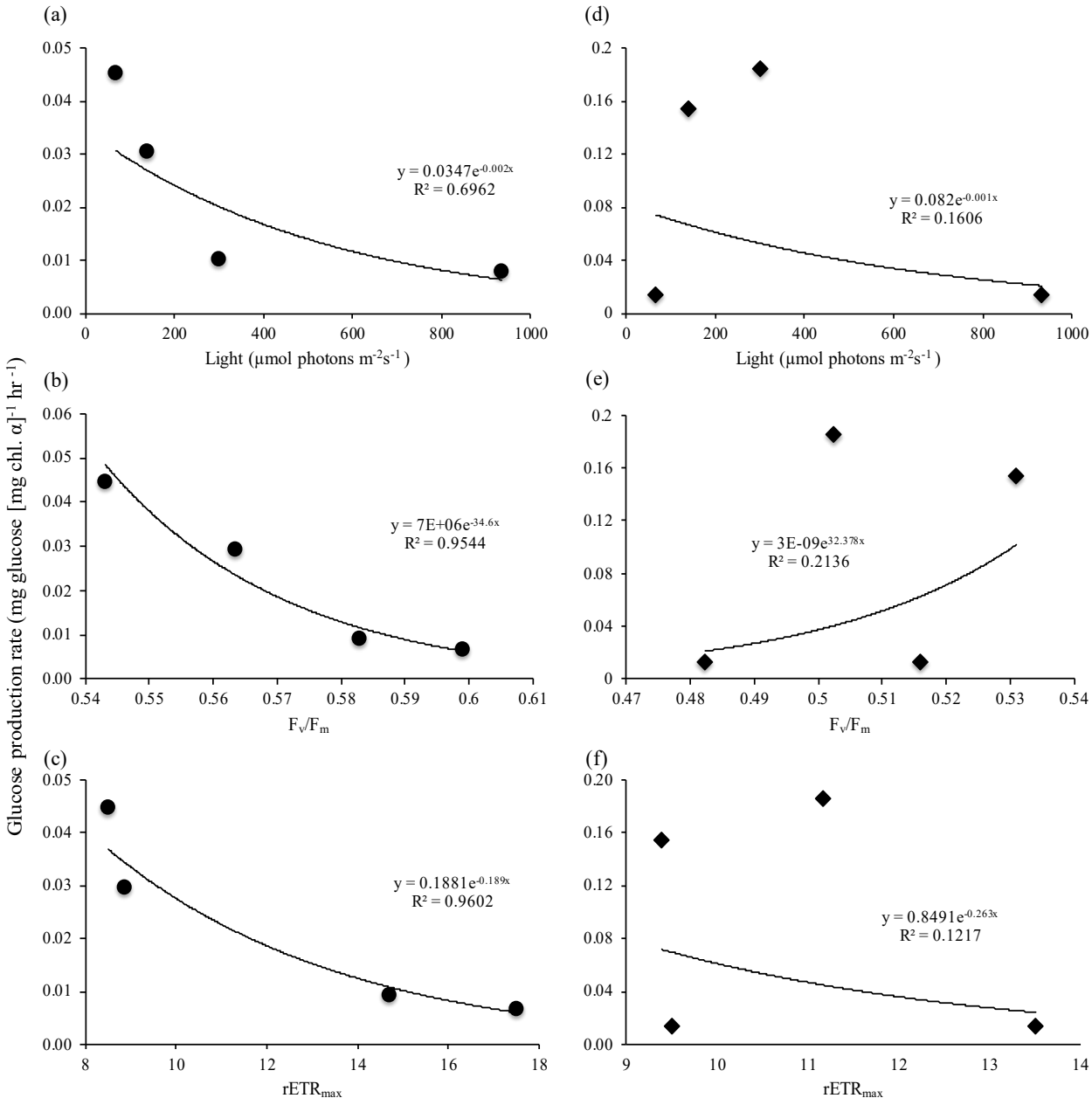
5.4.2.1. H. coffeaefirmis

The addition of an antibiotic significantly reduced the rate of bacterial glucose consumption in cultures of *H. coffeaefirmis* (Table 5.4.1). Importantly, there was no significant difference in the rate of glucose production by *H. coffeaefirmis* in cultures with and without the antibiotic (p-value= 0.191, F= 2.172, df=1). The antibiotic did have a significant impact on F_v/F_m (P-value= 0.00692, F= 16.205, df= 1), but not on $rETR_{max}$ (P-value= 0.561, F= 0.3785, df= 1). There were significant negative correlations between the rate of glucose production and light, F_v/F_m, and $rETR_{max}$ in the treatment without the antibiotic (Fig. 5.4.6 a-c). In the spiked treatment, the rate of glucose production only increased with increasing F_v/F_m but decreased with increasing light intensity and $rETR_{max}$ (Fig. 5.4.6 d-f).

Table 5.4.1. Rate of bacteria consumption in the non-antibiotic and antibiotic treatments for *H. coffeaeformis*, *N. menisculus*, and *N. longissima*, with ANOVA results showing the significant difference between treatments for each strain, where **bold** indicating significance.

Strains	Rate of consumption (mg glucose [mg chl.*a*]$^{-1}$hr^{-1}) (Antibiotic absence)	Rate of consumption (mg glucose [mg chl.*a*]$^{-1}$hr^{-1}) (Antibiotic presence)	df	F-values	P-values
H. coffeaeformis	0.0227	0.0206	1	2.32x10^{30}	**2x10^{-16}**
N. menisculus	0.140	0	1	2.03 x10^{32}	**2x10^{-16}**
N. longissima	0.226	0.0394	1	6.38 x10^{31}	**2x10^{-16}**

1993



1994

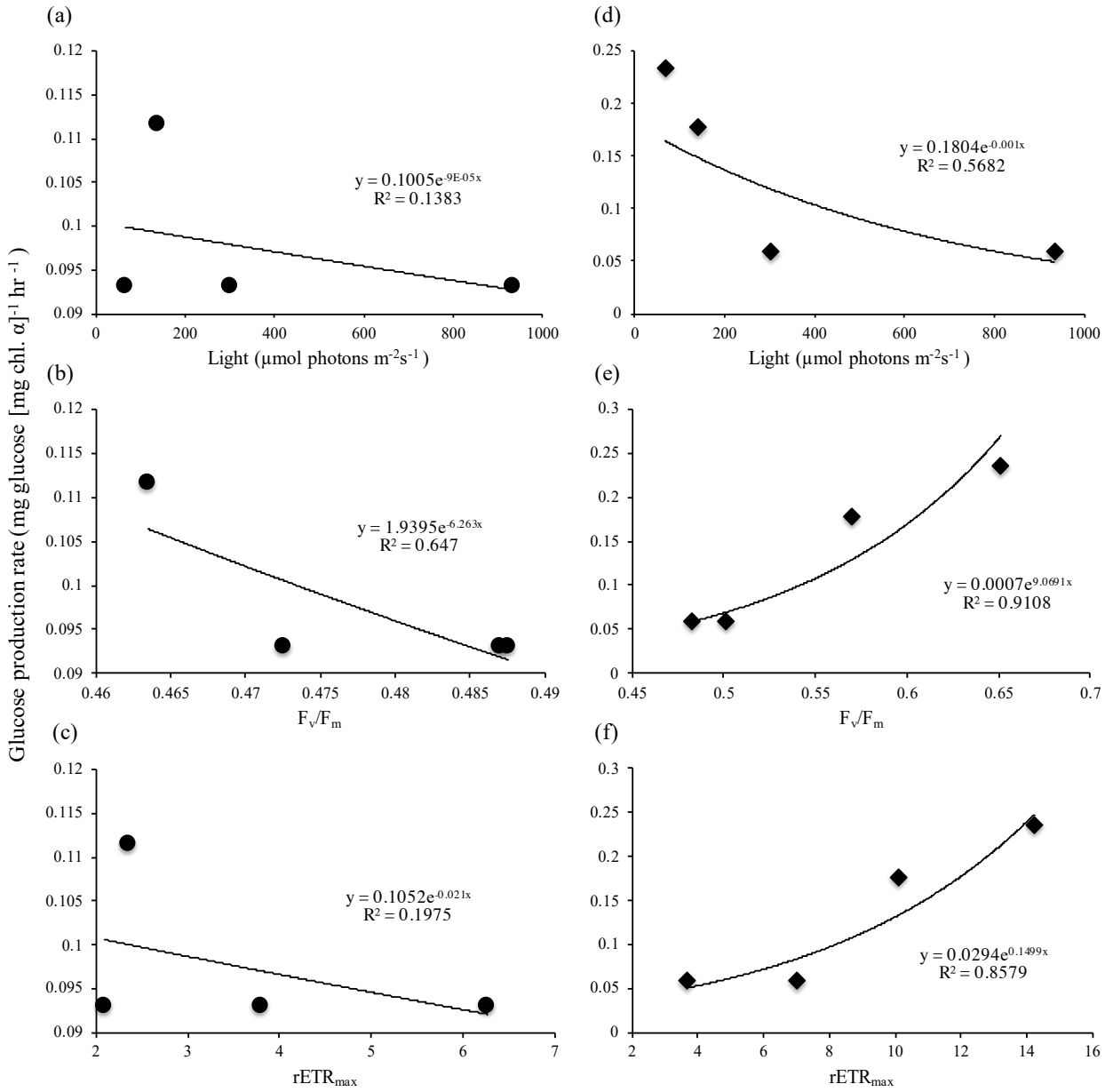
Figure 5.4.6. The relationship between the rate of glucose production and light, F_v/F_m, and $rETR_{max}$ for *A. coffeaeformis* culture in the absence of antibiotic [(a), (b), (c)] and in the presence of antibiotic [(d), (e), (f)] media.

5.4.2.2. N. menisculus

The rate of bacterial consumption in the *N. menisculus* culture ceased completely in cultures spiked with antibiotic (Table 5.4.1). There was no significant difference in the rate of glucose production by *N. menisculus* with respect to treatment (p-value= 0.468, F= 0.601, df=1). Furthermore, the antibiotic did not influence the photosynthetic performance of *N. menisculus* with respect to either F_v/F_m (P-value= 0.104, F= 3.658, df= 1) or $rETR_{max}$ (P-value= 0.0798, F= 4.4367, df= 1). Significant correlations were observed between the rate of glucose production in the presence of the antibiotic and F_v/F_m and $rETR_{max}$; production decreased with increasing light intensiy (Fig. 5.4.7 d), and decreased with increasing F_v/F_m, and $rETR_{max}$ (Fig. 5.4.7 e-f).

5.3.2.3. N. longissima

The rate of bacterial consumption also declined significantly in the *N. longissima* cultures treated with antibiotic (Table 5.4.1). There was no significant difference in the rate of glucose production between treatments (p-value= 0.828, F= 0.0513, df=1). The antibiotic influenced F_v/F_m (P-value= 0.0280, F= 8.299, df= 1), but not $rETR_{max}$ (P-value= 0.222, F= 1.858, df= 1). Significant correlations were observed between the rate of glucose production in the presence of antibiotic and light, F_v/F_m, and $rETR_{max}$; production decreased with increasing light intensities (Fig. 5.4.8 d), and decreased with increasing F_v/F_m, and $rETR_{max}$ (Fig. 5.4.8 e-f).



2016

2017 Figure 5.4.7. The relationship between the rate of glucose production and light, F_v/F_m, and
2018 $rETR_{max}$ for *N. menisculus* culture in the absence of antibiotic [(a), (b), (c)] and in the presence
2019 of antibiotic [(d), (e), (f)] media.

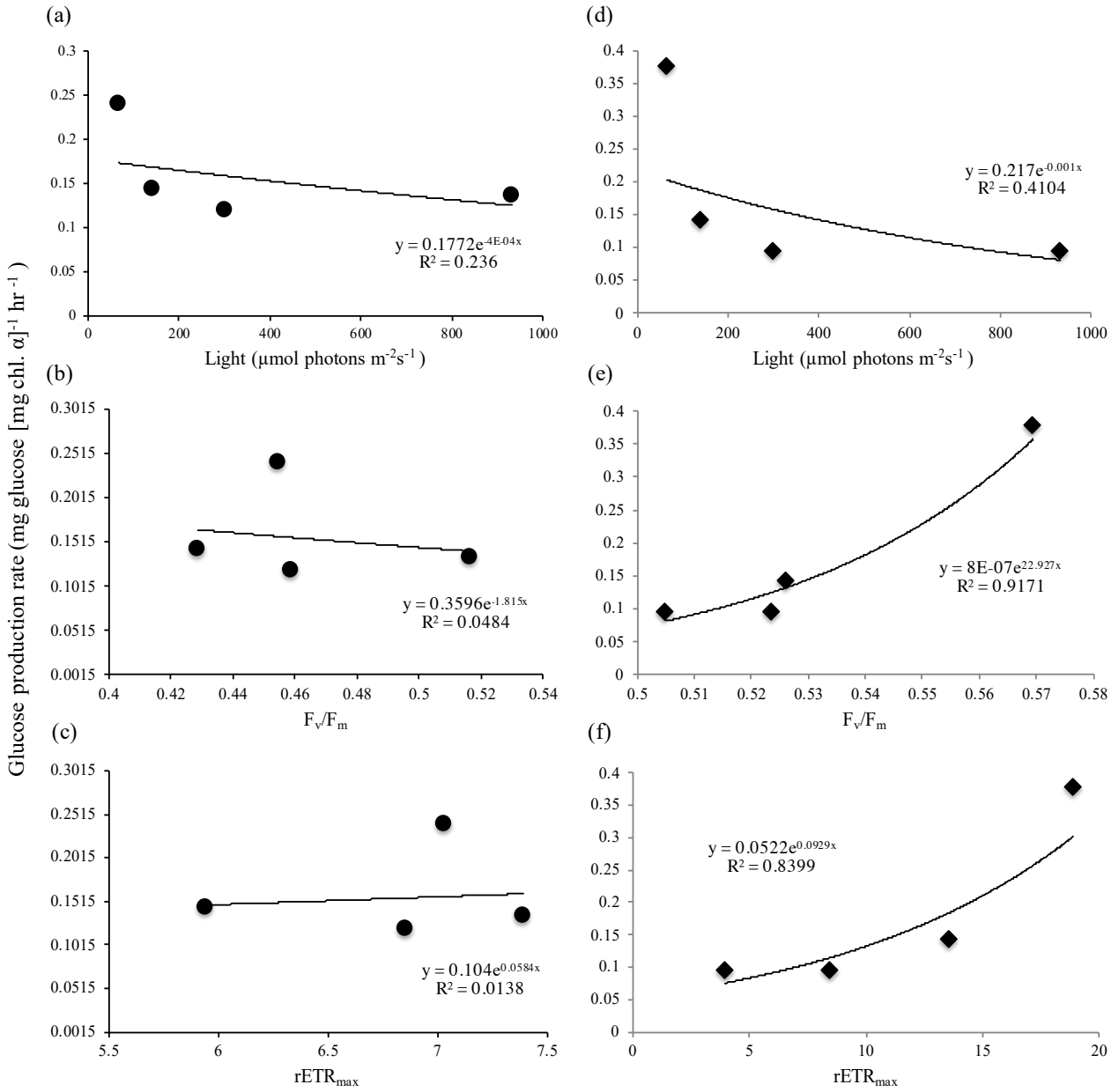


Figure 5.4.8. The relationship between the rate of glucose production and light, F_v/F_m, and $rETR_{max}$ for *N. longissima* culture in the absence of antibiotic [(a), (b), (c)] and in the presence of antibiotic [(d), (e), (f)] media.

5.4.3. MPB of Natural Sediments

In addition to the single-species experiments, the rate of glucose production and consumption was also measured in mixed-species cultures collected from the same sampling site. The rates of glucose production by benthic communities collected from the natural sediments were relatively low compared to the monocultures, and this trend was evident in nutrient and antibiotic manipulations (Fig. 5.4.9 and Fig. 5.4.10).

5.4.3.1. Nutrient treatment

Maximum glucose production in the mixed MPB communities varied from 0.0241 mg glucose [mg chl.*a*] $^{-1}$ hr^{-1} (nutrient-replete) to 0.0499 mg glucose [mg chl.*a*] $^{-1}$ hr^{-1} (nutrient-limited); lowest rates of glucose production were 0.0132 glucose [mg chl. *a*] $^{-1}$ hr^{-1} (nutrient-replete) and 0.0194 glucose [mg chl.*a*] $^{-1}$ hr^{-1} (nutrient-limited). The rates of production did not differ significantly (p=value 0.137, F= 2.952, df= 1). Nutrient concentration had a significant effect on F_vF_m but not on $rETR_{max}$ (p-value= 0.00581, F= 17.483, df= 1, p-value= 0.884, F= 0.0234, df= 1, respectively) (Fig. 5.4.9). There were significant correlations between the rate of glucose production and light, F_v/F_m, and $rETR_{max}$ in the nutrient-saturated experiments, as it increased with increasing light (Fig. 5.4.9 a) and decreased with increasing F_v/F_m (Fig. 5.4.9 b), and $rETR_{max}$ (Fig. 5.4.9 c). In the nutrient-limited experiments, similar trend was observed with an increased rate of glucose production with increasing light intensities (Fig 3.9 d) and decreased with increasing F_v/F_m (Fig. 5.4.9 e) and $rETR_{max}$ (Fig. 5.4.9 f).

5.4.3.2. Bacteria treatment

Bacterial consumption in the mixed MPB communities was significantly reduced in the antibiotic treatment relative to the control (P-value= $2x10^{-16}$, F= $4.02x10^{30}$, df= 1). There was no difference in the rate of glucose production (p-value= 0.0810, F= 4.388, df=1), F_v/F_m (P=value= 0.263, F= 1.524, df=1) or rETRmax (P-value= 0.236, F= 1.732, df=1) with respect to treatment. Glucose production was correlated with light, F_v/F_m, and $rETR_{max}$ in the antibiotic treatment; production increased with increasing light (Fig.5.4.10 d) and decreased with increasing F_v/F_m (Fig. 5.4.10 e) and $rETR_{max}$ (Fig. 5.4.10 f).

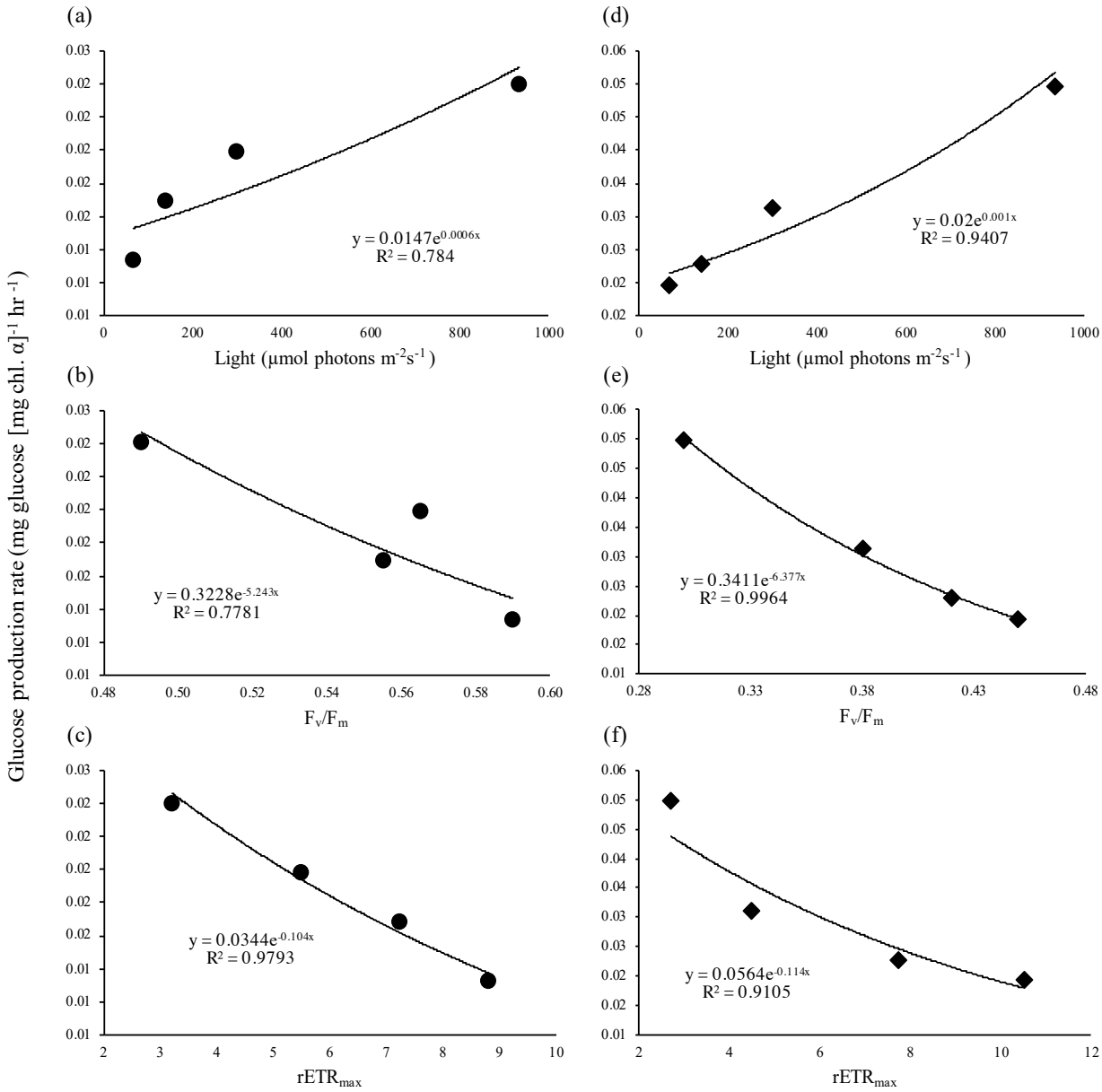
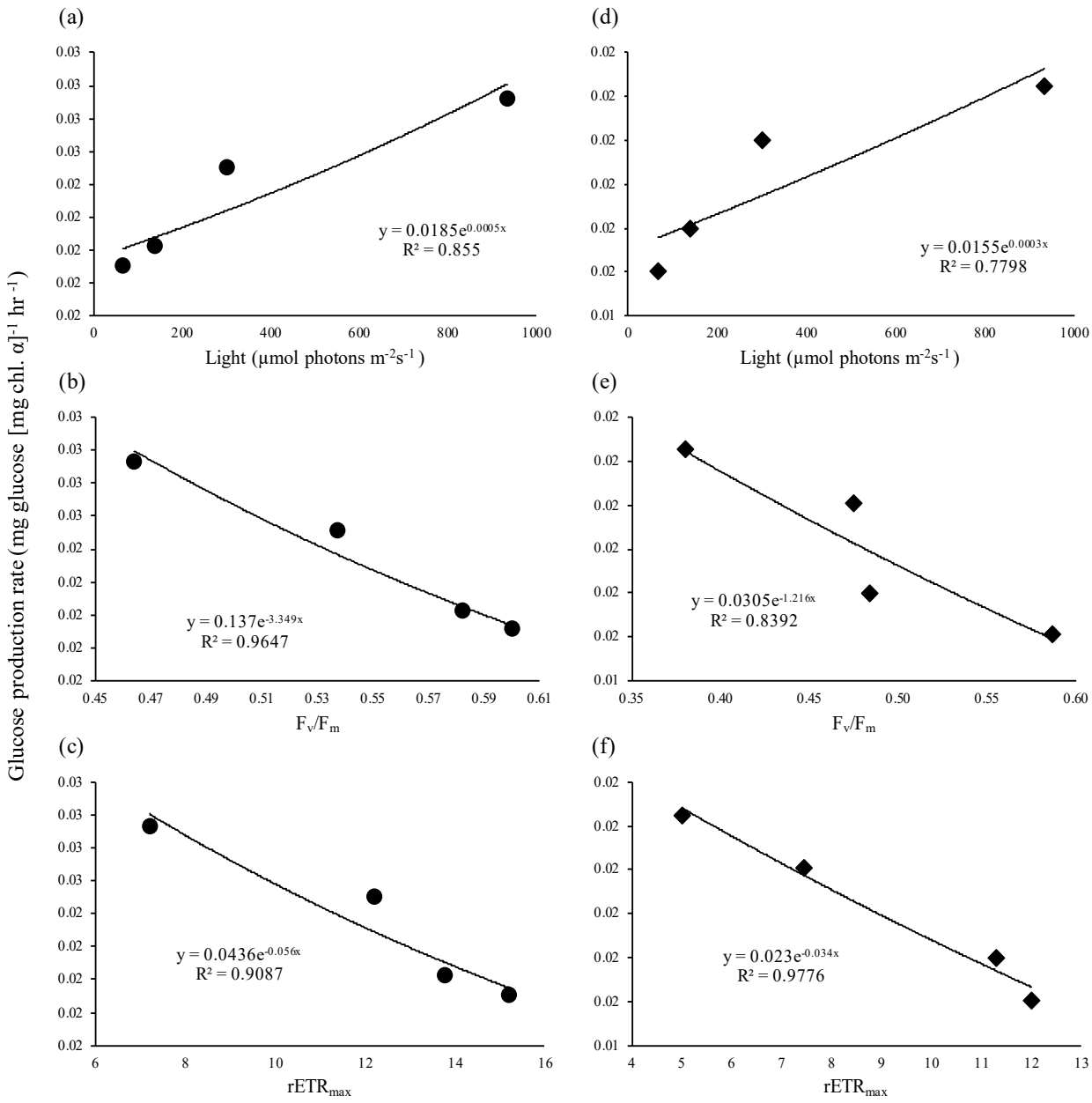


Figure 5.4.9. The relationship between the rate of glucose production and light, F_v/F_m, and $rETR_{max}$ by MPB of natural sediment in nutrient saturated [(a), (b), (c)] and nutrient limited [(d), (e), (f)] media.



2055

2056 Figure 5.4.10. The relationship between the rate of glucose production and light, F_v/F_m, and
2057 $rETR_{max}$ for MPB from natural sediments in the absence of antibiotic [(a), (b), (c)] and in the
2058 presence of antibiotic [(d), (e), (f)] media.

5.5. Discussion

This study documents the first real-time measurements of MPB glucose production using a novel biosensor. Previous studies have shown that glucose can account for up to 90% of the total extracellular carbohydrates produced by MPB (Staats et al., 1999, Goto et al., 2001, Bhosle et al., 1995). Importantly, up to 60% of the assimilated carbon can be excreted into the water column within 1 hour (Smith and Underwood, 2000). The rates of glucose production observed in this study demonstrated that MPB are influenced by irradiance leading to photosynthetic overflow.

Nutrient availability can have a significant effect on MPB performance in the intertidal (Christian et al., 2001; Cook et al., 2004; Cook and Røy, 2006; Cook et al., 2007; Mueller et al., 2016; Underwood and Provot, 2000). Interestingly, nutrient limitation did not greatly impact glucose production in the three species examined in this study. This is comparable with the findings of Cook et al. (2007), who carried out mesocosm experiments on sublittoral sands in Germany, and showed that MPB communities are capable of sustaining high rates of photosynthesis despite nutrient limitation. Conversely, in other studies, species such as *Cylindrotheca closerium* and *Navicula subinflata* have been greatly influenced by the concentration of available nutrients (Bhosle et al., 1995; Staats et al., 2000b). Canion et al. (2014) also supported the notion that nutrient concentration is one of the main factors in determining the extracellular carbohydrate concentrations in the sediment. MPB biomass generally correlates with nutrient levels in the water column and extracellular carbohydrates, hence any increase in MPB biomass will result in an increase in extracellular carbohydrates (Bellinger et al., 2010). The photosynthetic performance of the single-species cultures was influenced by nutrients, but not the mixed MPB assemblages. Other authors have shown that acclimation of biofilms to a particular light climate correlates with the nutrient status (Underwood, 2002). In this study, the observed F_v/F_m values were generally high, but were highest in nutrient-limited cultures. The MPB collected from the natural sediment also showed that glucose production was not influenced by nutrient concentrations. In comparison to the single-species cultures, the rate of glucose production by the MPB cultures collected from the natural sediment was relatively low.

Diatom mucilage constitutes a valuable carbon source for heterotrophic bacteria (Decho and Lopez, 1993). In the cultures with no antibiotics, the glucose concentration decreased significantly as soon as the light was switched off, a trend that was not mirrored in the spiked

cultures. This infers that glucose was utilised as a substrate by bacteria and is comparable to the results of Friend et al. (2003), who reported higher erosion thresholds during the night compared to the dark in the intertidal, despite the higher EPS concentrations secreted under illumination (Smith and Underwood, 2000; de Brouwer and Stal, 2001). During bacterial degradation, the polymers are subjected to conformational changes and chemical alterations that might further impact their binding capabilities. Therefore, light-produced, loosely attached EPS mainly comprising glucose is a relatively easy target for prokaryotes (Hofmann et al., 2009).

While bacterial consumption completely ceased in *N. menisculus* cultures spiked with the antibiotic, and was minimal in *N. longissima*, this was not the case for *H. coffeaefirmis* cultures. It appears likely that *H. coffeaefirmis* itself consumes photosynthate in the dark. In a previous study, Staats et al. (2000a) observed that *C. closterium* decomposed its own extracellular carbohydrates in the dark. Interestingly, not all MPB consume extracellular carbohydrate in the dark, as evidenced in this study with cultures of *N. menisculus* and *N. longissimi*.

Experiments on the natural sediment cores in this study showed that bacterial activity is more pronounced compared to the cells present in single strain algal cultures, as the rates of consumption were a lot higher. This highlights the more complicated interactions that occur between mixed species of MPB and mixed species of bacteria in the natural environment (Smith and Underwood, 2000). In the naturally collected MPB communities, the rate of glucose consumption was not completely impaired by the use of antibiotics either. This is mainly due to the complexity of the microphytobenthic communities in the sediment. As different species of bacteria live in the intertidal zone, killing all bacteria with one form of antibiotic is not possible. In addition to the multiple bacterial species, the presence of mixed MPB communities was another reason that glucose levels remained high. As was observed in the experiment with *H, coffeaefirmis*, glucose consumption was not completely terminated after the light was switched off. Therefore, not all species of MPB consume extracellular carbohydrates during the dark, or not immediately.

5.6. Conclusion

Modified glucose biosensors were capable of accurately measuring the real-time MPB production of algal exudates in marine sediment. Variation in nutrient concentration did not have a significant effect on production but did lead to contrasting rates of photosynthetic

performance. The bacterial consumption experiments showed that glucose was consumed not only by bacteria, but also some MPB species in the dark. The mixed-species experiments further showed that there are additional challenges in measuring real-time changes in the glucose production with complex microbial assemblages.

Chapter 6: Conclusion

In this study (Chapter 2 and 3) it was found that extracellular carbohydrates, were produced in response to high irradiances and/or low nutrient levels either as either photosynthetic overflow, or in the form of mucilage for vertical migration or other EPS.

The composition of the extracellular carbohydrate in the sediment, which was estimated using the TPTZ assay was found to be dominated by monosaccharides, produced during the emersion period (Chapter 2 and 3). A further, more specific study of the monosaccharide component (using HPLC), identified glucose as the major component (Chapter 4). An additional study also identified real-time fluctuations in glucose production and found that the glucose was produced as photosynthetic overflow (Chapter 5).

This study focused on the performance of microphytobenthos (MPB), particularly extracellular carbohydrate production, in response to environmental variables. Intertidal zones are highly dynamic, hence MPB need to respond efficiently to the rapid changes in their surrounding environment. Extracellular carbohydrate production is important both photosynthetically and behaviorally. MPB mainly produce extracellular carbohydrates for vertical migration, i.e. for positioning themselves at the optimum position in the sediment, as well as to migrate away from photodamaging irradiance. In addition to its role in vertical migration, extracellular carbohydrates are also produced as a photosynthetic overflow product when MPB are experiencing extreme environmental conditions, such as excessive irradiance, extreme sediment temperature (i.e. > 35°C or freezing temperature), nutrient-limited condition, and fluctuations in salinity. In spite of their small size, the importance of MPB should not be underestimated. This study not only investigated the MPB communities from southern temperate intertidal flats, but also covered those of tropical regions.

The study initially studied the MPB in a coastal area of southeast Tasmania. Results showed that it was well adapted to its changing environment, as it was only affected by salinity. Two study sites were selected. Penna Beach, with a sediment composition of sandy mud has much greater concentrations of extracellular carbohydrates than those of Kings Beach, which was composed of sandy sediment. This differences in grain size resulted in different species abundances and diversities. Additionally, the coarser sediment composition of Kings Beach may have permitted the MPB-produced carbohydrates to be more easily washed away by wave action. This study has also investigated the annual cycle of MPB performance at both sampling

sites. Significant differences between seasons were found in this study, with spring and summer having higher chl.*a*-normalised extracellular carbohydrate concentrations than those of winter and autumn. Additionally, the concentrations of chl.*a*-normalised monosaccharide (MCHO) were significantly higher than the concentrations of chl.*a*-normalised polysaccharide (PCHO). This is thought to be because PCHO was produced for vertical migration, while MCHO was produced as a photosynthetic overflow product, in response to unfavored environmental conditions.

Microphytobenthic photosynthesis and extracellular carbohydrate production were also being investigated in a tropical region, Penang, Malaysia. MPB biomass and diversity in Penang were strongly influenced by sediment temperature, irradiance, and salinity. As sampling was carried out during the raining period of dry season, there was high precipitation during most of the sampling periods. The irradiance fluctuated significantly due to the dense cloud cover and both sediment temperature and salinity were affected by the cool freshwater introduced into the intertidal area.

Glucose is usually the major sugar component of the total extracellular carbohydrate production. Therefore, real-time changes in concentration are an important factor in gaining a better understanding of how MPB respond to their environment. This study was able to achieve this real-time-changes measurements, by using an innovative glucose biosensor protocol. This technology does not only allow the instantaneous measurement of glucose production in response to the changing environmental variables, it also allows the determination of glucose consumption by bacteria. In this study, the biosensors successfully measured the instantaneous changes in glucose concentrations in response to different experimental set ups, on both single and mixed cultures. In the experiments of single-species cultures with varying nutrient concentrations, the rate of glucose production did not vary significantly between treatments, but it increased with increased irradiance, as the glucose was most likely produced as photosynthetic overflow in response to the saturating irradiance. In the experiments examining bacterial consumption of glucose, glucose consumption in the dark mostly ceased in the presence of an antibiotic. However, in some cultures, such as *H. coffeaefirmis*, a small continuing drop is explained by heterotrophic glucose consumption by MPB.

In addition to single-species cultures, mixed MPB communities were also examined, by collecting the natural sediment directly from the sampling site. Results from these communities showed that the rate of production was lower than that of single-species cultures. This is

thought to be due to competition between species in the mixed communities. It may also be due to the higher complexity in the mixed communities which would have included species that had higher heterotrophic uptakes, thus reducing the 'apparent' rate of glucose production in the sediment. The application of glucose biosensors to measuring glucose production of the mixed MPB communities raises promising expectations of applying this technology to field studies, also to targeting different sugars, such as galactose, rhamnose, fucose, and xylose.

Qualitative analysis was undertaken to characterize the MPB extracellular carbohydrate composition at these sites. High performance liquid chromatography mass spectrometry (HPLC-MS) was used for these analyses to determine the concentrations of selected sugars within the extracellular carbohydrate pool produced by MPB. Among the targeted sugars (rhamnose, arabinose, xylose, mannose, galactose, and glucose), glucose contributed the greatest proportion (>80%) of the total extracellular carbohydrate. The chl.*a*-normalised glucose concentrations at Penna Beach were significantly higher than that of Kings Beach. This is thought to be mainly due to differences in sediment composition. In addition to greater mobility, the coarser sediment at Kings Beach had deeper light penetration, so MPB did not need to produce additional extracellular carbohydrate for vertical migration., The glucose was mainly produced as a photosynthetic overflow product, especially during spring, summer and autumn, when irradiances and sediment temperatures were significantly higher. It has been found that bacterial DOC consumption is vital to the efficient working of the microbial loop. Hence, the concentrations of different sugars at different depths was also investigated in order to determine the significance of bacterial activity on the concentration of extracellular carbohydrates. The elevated glucose concentrations may have been due to the high bacterial activity. Glucose is the preferred monosaccharide to be consumed by bacteria. Thus, it is likely that hydrolisation of larger monomer extracellular carbohydrates into smaller molecule sugars such as glucose will be undertaken by the bacteria. Additionally, previous studies have also found that β- and α-glucosidase activities were high during the low-tide period. The chl.*a*-normalised glucose concentrations did not vary significantly between sampling depths at either sampling site. This is probably because the elevated chl.*a*-normalised glucose production at the sediment surface was caused by photosynthetic overflow, while the high chl.*a*-normalised glucose concentrations at greater depths was due to the breakdown of larger molecule to extracellular carbohydrates into glucose by the exo-enzyme produced by the bacteria.

In conclusion, MPB from different regions undertake different mechanisms in response to their dynamic environment Changing environmental conditions had the greatest effect on MPB of tropical regions, as continuous increased in irradiance and sediment temperature may have caused the threshold they could withstand to be breached (>2000 (μmol photon $m^{-2}s^{-1}$ and >35°C), which results in photodamage to the cells. Additionally, glucose biosensors have produced promising results in this study, which have opened up opportunities for future field studies, perhaps targeting alternative carbohydrate options such as rhamnose, arabinose, xylose, mannose, and galactose.

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USE OF GLUCOSE BIOSENSORS TO MEASURE EXTRACELLULAR GLUCOSE EXUDATION BY INTERTIDAL MICROPHYTOBENTHOS IN SOUTHERN TASMANIA[1]

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Micro glucose biosensors were used to measure net extracellular glucose produced by natural microphytobenthos and three diatom cultures (*Amphora coffeaeformis*, *Navicula menisculus*, *Nitzschia longissima*) from southern Tasmania, Australia. They were exposed to a light gradient in either nutrient-replete or nutrient-limiting conditions. Glucose exudation in the natural communities increased with increased light but the response in the cultures was variable. Similarly, nutrient-replete conditions elicited lower rates of glucose exudation in the natural communities but produced variable species-specific responses in the cultures. Increased glucose exudation mostly correlated with a reduction in maximum quantum yield (F_v/F_m). The same trend was observed in the natural communities for relative maximum electron transfer rates ($rETR_{max}$) but responses in the cultures were again variable and species-specific. Responses of the three species to increased light and nutrient deficiency were variable, although glucose exudation, F_v/F_m and $rETR_{max}$ was mostly lower in the nutrient-limited media. In a second set of experiments species/communities were treated with/without antibiotics. In the dark, glucose concentrations in treatments with antibiotics remained unchanged, while in those with bacteria, it fell rapidly. In the sediment communities, glucose consumption in the dark was ~25% the rate of exudation at the highest light level. In culture, exudation rates were up to 100% greater than those with active bacteria. Rates of glucose consumption in the dark in the antibiotic–treated samples were negligible and up to 10^4 times lower than those with active bacteria. These results demonstrate the important role extracellular glucose exudation has on maintaining an active microbial loop.

Key index words: **bacteria; biosensor; glucose; light; microphytobenthos; MPB; nutrients**

Abbreviations: **Chl *a*, chlorophyll *a*; DOC, dissolved organic carbon; EPS, extracellular polymeric substances; F_v/F_m, photosynthetic efficiency (maximum quantum yield of fluorescence); MPB, microphytobenthos; rETR, relative electron transfer rate; RLC, rapid light curve**

Primary production in near shore coastal and estuarine ecosystems is primarily generated by phytoplankton and biofilms of photosynthetic benthic microalgae (microphytobenthos, MPB) with smaller contributions from other sources. MPB is the biofilm of photosynthetic microorganisms that grows on the bottom or in the intertidal zone of these shallow ecosystems. At depths within the euphotic zone, typically 5–20 m below the surface, it regularly contributes between 25% and 50% of the total annual primary productivity (Cahoon 1999, Underwood and Kromkamp 1999). Within the euphotic zone, MPB biomass can exceed 500 mg chl $a \cdot m^{-2}$ (Underwood 2010), which is often greater than the depth-integrated phytoplankton biomass. Furthermore, it is the primary food source for many benthic invertebrates (Kang et al. 2007, Lebreton et al. 2011), which then sustains substantial fish and wading bird populations.

Extracellular carbohydrate exudation is widespread in photosynthetic biofilms, being produced by both bacteria and micro algal cells. It has been found to have a number of functions including cell attachment, motility, and protection (Hoagland et al. 1993, Cooksey and Wigglesworth-Cooksey 1995, Underwood and Kromkamp 1999, Staats et al. 2000, Cook et al. 2007). Much of the extracellular polymeric substances (EPS) is mucilaginous and is comprised of polysaccharides, uronic acids, and sulfated sugars (Underwood and Paterson 2003, Bellinger et al. 2009, Oakes et al. 2010). Dissolved organic carbon (DOC) production and export by microalgae is a core requirement of the microbial food web and is taken up rapidly by both heterotrophic bacteria and the microalgae themselves. Extracellular release of DOC from microalgae is mostly from either photosynthetic overflow, whereby

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